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Influence of Yolk on Mitotic Rate in Untreated and X-Rayed Grasshopper Neuroblasts *in vitro*.* (22018)

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(Introduced by Alexander Hollaender.)
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The 14-day-old embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) has been extensively used by Carlson, Gaulden, and coworkers for *in vitro* studies of its large, rapidly dividing neuroblasts. In the course of some experiments requiring rapid preparation of hanging-drop cultures of irradiated embryos, the small amount of grasshopper egg yolk usually added to the artificial medium (1) in the cultures was omitted to speed operations. The recovery of the neuroblasts in these cultures from radiation-induced mitotic inhibition was greatly retarded. The present report describes a detailed examination of the influence of yolk on mitotic rate in untreated neuroblasts and on recovery from mitotic inhibition in X-rayed neuroblasts. A positive influence was found in each case.

Materials and methods. In all experiments embryos were dissected and made into hanging-drop preparations for *in vitro* observations as described by Gaulden, Nix, and Moshman (2), with two exceptions. Following separation from their membranes and removal of their appendages (hereafter referred to as "dissection"), the embryos were left surrounded by yolk in a dish of artificial culture medium until they were mounted in hanging-drop cultures. Varying amounts of yolk, or no yolk, were added to the cultures. For study of the influence of yolk on mitotic rate of untreated embryos, 4 different amounts were tested, namely, approximately 0, ⅛, ¼, and ½ the amount present in an egg. Amounts of yolk greater than ½ were not used because they increased greatly the osmotic pressure of the culture drop resulting in a change in mitotic rate(3). With the amounts of yolk used in the present experiments, no changes in osmotic pressure—as determined by the visible appearance of cells and chromatin—were observed except possibly during the later counting periods in embryos mounted with ½ yolk. A given amount of yolk can be obtained by placing a 14-day-old egg (Fig. 1) in a dish of culture medium,

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cutting off the posterior tip of the egg, and gently pressing the embryo and yolk out of the chorion. If this operation is performed carefully, the yolk comes out in the form of a cylinder with the embryo lying on top at one end. The embryo and its membranes can be easily discarded without disturbing the yolk. The cylinder of yolk can then be cut into portions of desired size. The anterior ⅛, ¼, or ½ of the yolk cylinder was used because its consistency was more uniform than that of the posterior portion. Currents in the medium around the yolk are kept at a minimum during these operations in order to prevent dispersal of the serosal fluid which surrounds the yolk. Twelve minutes after dissection each embryo was mounted on a cover glass, ventral surface down, in a drop of culture medium to which was added a given amount of yolk or no yolk. The total amount of culture material in the hanging-drop was equal to 5-10 μl of fluid. The cover glass was inverted and sealed over a depression slide with thick mineral oil. The volume of the depressions used varied from 0.0531 to 0.0594 ml. Twenty-one minutes after the embryos had been dissected the hanging-drop preparations were placed on the stage of a microscope, the lower portion of which was enclosed in an incubator maintained at 38° ± 1°C. Observations on the neuroblasts, which lie just beneath the cover glass in these preparations and which are in all stages of mitosis, were begun 1 minute later with the oil immersion lens and bright-field illumination. Light from the microscope lamp was passed through a 5% solution of copper sulfate to remove heat. For study of the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic injury, embryos were exposed *in vivo* to 64 r of X rays at room temperature (25° ± 2°C). A General Electric Maxitron 250-kvp unit was used at 125 kvp and 15 ma with 3.5 mm Al filtration and 54.5 cm target distance. The dose rate was 32 r/minute. Embryos were dissected 18 minutes after irradiation and mounted 2 minutes later. In preparation of the hanging-drop cultures, the X-rayed embryos were mounted, half with ¼ yolk and half without yolk. Untreated control embryos were similarly mounted. Twenty-one minutes after irradiation, the hanging-drop cultures of both treated and control embryos were placed in the incubator at 38° ± 1°C and observations begun 1 minute later.

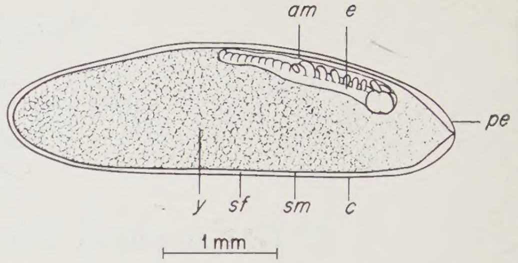

FIG. 1. *Camera lucida* sketch of 14-day-old egg of grasshopper, *Chortophaga viridifasciata*. *am*, amnionic membrane; *c*, chorion and closely adhering cuticle layers; *e*, embryo; *pe*, posterior end of egg; *sf*, serosal fluid; *sm*, serosal membrane; *y*, yolk.

The method for determining mitotic rate is as follows. Approximately 140 neuroblasts per embryo (all neuroblasts in the 3 thoracic segments) are examined at 16 22-minute intervals and the number of neuroblasts in mid-mitosis (prometaphase, metaphase, and anaphase) are recorded. Such counts provide a valid determination of the number of cells going through mitosis in a given period of time because the duration of mid-mitosis at 38°C is 22 minutes under the conditions of the present experiments. The short interval between observation periods limits the number of embryos that can be examined at one time. Thus the data for each experimental condition represent a compilation of data obtained from several sets of embryos studied on several successive days. In the experiments to test the influence of yolk on untreated mitosis, 2 embryos with each of the 3 amounts of yolk and 2 with no yolk were prepared for study at a time. In the X-ray experiments, 4 X-rayed and 4 untreated embryos, 2 with and 2 without yolk in each case, were studied at a time.

Results. Results of the experiments designed to determine the influence of yolk on mitotic rate of untreated neuroblasts are given in Fig. 2 as the average number of mid-mitotic cells per embryo plotted against time after dissection. An analysis of variance test† of

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† The authors are indebted to Dr. A. W. Kimball and Mr. George Atta for the statistical analyses of the data.

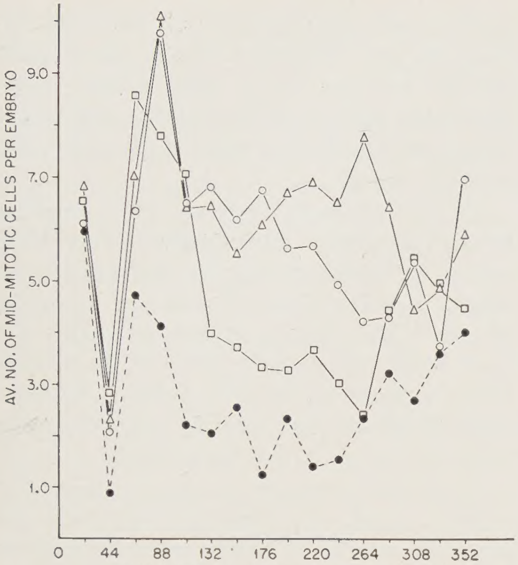

FIG. 2. Effects of varying amounts of yolk from a grasshopper egg on mitotic rate of untreated neuroblasts. ●, no yolk (8 embryos); □, ⅛ yolk (9 embryos); △, ¼ yolk (8 embryos); ○, ½ yolk (7 embryos).

the raw data revealed that the shapes of the curves are essentially the same but that their over-all heights are different at the 0.1% level of significance. The number of mid-mitotic cells in the no-yolk cultures as compared with the yolk cultures at the 44- and 66-minute counts (Fig. 2) was significantly lower only at the 5-10% level of probability. This cannot be considered a statistically significant difference, but it does indicate an early trend toward low mitotic activity in the no-yolk cultures that reaches significant proportions by the 88-minute count. In other words, the decrease in number of cells that can complete mitosis in the absence of nutritive yolk becomes evident soon after the cells are separated from the yolk.

The drop in the frequency of mid-mitotic cells at the 44-minute count followed by a rise at the 66- and/or 88-minute counts occurred in all 4 types of culture preparations. Exploratory experiments revealed that this fall and rise of activity results from a brief inhibition of mitotic progress of cells which enter or are in middle and late prophase when cultures are prepared. This inhibition appears to be caused partly by temperature shock that results when cultures are transferred from room temperature to 38°C and partly by the slight adjustments in cellular osmotic pressure which occur immediately following the change from *in vivo* to *in vitro* conditions.

In Fig. 3 is shown the relation between amount of yolk in the culture preparation to the mean number of cells completing mitosis in 352 minutes. It can be seen that the frequency of cells going through mitosis increased as the quantity of yolk increased up to ¼‡. The mean mitotic frequency in cultures containing ½ yolk was a little lower than that in the ¼-yolk cultures. This is probably caused by adverse effects of the slightly higher osmotic pressure that develops in the medium of the ½-yolk cultures about 3.5 hours after they are prepared. The cause of this increased osmotic pressure has not yet been determined. In cultures made up with amounts of yolk greater than ½, the increased osmotic pressure becomes manifest very soon after their preparation. For this reason larger amounts of yolk were not used in the present study.

Results of the experiments designed to de-

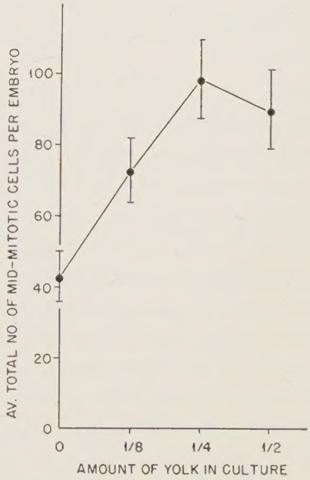

FIG. 3. Total number of mid-mitotic neuroblasts per embryo as function of amount of yolk in culture drop. The interval about each point represents the region within the 95% confidence limits.

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‡ It should be noted that an amount of yolk approximately equal to ¼ that in an egg has been routinely used in preparing hanging-drop cultures in all previous work on grasshopper neuroblasts.

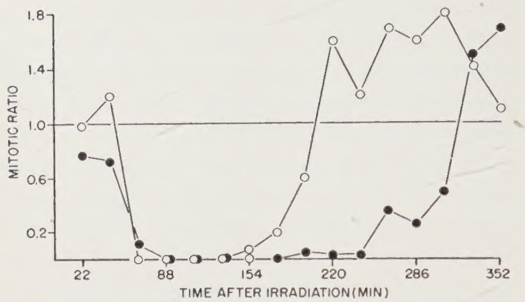

FIG. 4. Effects of yolk on recovery of neuroblasts from X-ray (64 r) induced injury. ○, mitotic ratio of X-rayed cells : control cells mounted with ¼ yolk; ●, mitotic ratio of X-rayed cells : control cells mounted without yolk. Data obtained from 8 embryos for each experimental condition.

termine the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic inhibition are given as mitotic ratios in Fig. 4. It can be readily seen that the duration of the inhibition period is shorter and the beginning and completion of recovery is considerably faster in the neuroblasts of embryos mounted in yolk than in those of embryos mounted without yolk. These differences are statistically significant at the 1% level as shown by an analysis of variance of the raw data. In other words, yolk greatly enhances the recovery of neuroblasts from X-ray-induced mitotic injury.

Discussion. Untreated neuroblast. The decrease in mitotic rate which is evident soon after neuroblasts are separated from yolk suggests that the neuroblast does not go through a given mitosis entirely on energy previously stored within the cell. The frequency of mid-mitotic cells in the no-yolk cultures reaches a statistically significant low level at the time (88-minute count) that the frequency in the yolk cultures is at its maximum (Fig. 2). This high frequency in yolk cultures is the result of recovery of middle and late prophase cells from a temporary inhibition induced by change from *in vivo* to *in vitro* conditions. It would appear, therefore, that the absence of yolk considerably affects the ability of middle and late prophase cells to complete mitosis.

Under optimum *in vitro* growth conditions, cells which were in early prophase and earlier stages or even in the previous mitosis would be expected to reach mid-mitosis at the 88-minute and successive counts.[§] Since the frequencies of mid-mitotic cells in the no-yolk cultures are very low at these counts, it appears that cells in early prophase and earlier stages are unable to complete mitosis in the absence of yolk.

Cells in stages from very late prophase through anaphase either require very little energy for their processes or they operate on energy stored within the cell during earlier phases. The second explanation seems the more likely, especially in view of the considerable amount of activity, such as chromosome movement and cleavage furrow formation, that occurs during these stages.

Several investigators are of the opinion that a cell is capable of completing mitosis without acquiring additional energy after the process has begun. Bullough(4,5) has data on mitosis in mouse epidermis which he interprets as evidence that these cells need considerable amounts of energy to initiate mitosis but very little energy to complete it. Swann(6,7) has proposed, on basis of his work with developing sea urchin eggs and that of Jacoby, *et al.* (8) on chick fibroblasts, that the energy necessary for a given mitosis is supplied by the "siphoning" of an energy "reservoir" which is stored during the previous mitosis. In other words, conditions which adversely affect the energy supply of the cell would not be detected until the subsequent division. The results herein reported suggest that in the grasshopper neuroblast at least the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis.

That the energy for division is derived from glycolysis seems a reasonable conclusion to be drawn from the results of several workers (9-12). Tipton and St. Amand(13) have shown, however, that glucose does not influence respiration of the 14-day-old embryo of the grasshopper *Chortophaga viridifasciata.* This is not surprising in view of the findings of Bodine(14) and Hill(15) that fat seems to be the main source of energy for grasshopper embryos of this age.

[§] These calculations are based on durations of the various mitotic phases as given by Carlson and Hollaender, *J. Cell. and Comp. Physiol.,* 1948, v31, 149.

Fischer(16) has found that the growth of chick myeloblasts and osteoblasts *in vitro* is dependent on several amino acids, cysteine being the most essential. Shaw(17) has identified 17 free amino acids in the 14-day-old egg of *Chortophaga*. However, since his analysis is based on entire contents of the egg, it is difficult to estimate the possible role of these amino acids in mitosis of the neuroblast.

Preliminary experiments in this laboratory indicate that a filtrate‖ of fresh grasshopper egg contents gives the same result as ¼ yolk. The filtrate contains miscroscopic globules of yellow "yolk" material but no other visible components.

Irradiated neuroblast. It is not known whether recovery from radiation-induced mitotic inhibition involves the replacement of some vital substance which was destroyed by radiation or the elimination of some substance which caused the inhibition(18). The pronounced positive influence of yolk on recovery of neuroblasts from radiation damage demonstrates that in this cell some extracellular metabolite or metabolites greatly enhance repair of mitotic damage. This result strikingly parallels that of Stapleton and coworkers(19,20,21) who have found that the addition of beef or yeast extracts, or amino acids to culture medium following irradiation causes considerable increases in the number of bacteria which survive the radiation. The amino acids in the grasshopper egg(17) may, therefore, be important factors in the recovery of the neuroblast from radiation damage.

The shapes of the curves in Fig. 4 suggest that the response of neuroblasts to 64 r of X rays in the absence of yolk differs from the response in presence of yolk only with respect to the duration of the inhibition period. The temporary retardation of cells in very early, early, middle and late prophase is mainly responsible for the inhibition period in the presence of yolk. Whether the absence of yolk affects recovery of cells in all these stages to the same extent or whether it differentially affects recovery of certain ones cannot be determined from the present data.

Summary. 1. The frequency of mid-mitotic neuroblasts in grasshopper embryos in hanging-drop cultures increases as the quantity of yolk in the culture is increased up to a quantity equivalent to ¼ that in an egg. The influence of yolk on mitosis in the untreated neuroblast is interpreted as indicating that, in this cell at least, the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis. 2. The pronounced positive effect of yolk on recovery of neuroblasts from radiation damage demonstrates that, in this cell, some extracellular metabolite or metabolites greatly enhance repair of mitotic damage.

‖ The extraembryonic contents of 14-day-old grasshopper eggs were mixed in 10 volumes of culture medium and passed through a fine Seitz filter. Culture medium rather than plain water was used because yolk and, in particular, serosal fluid contain much protein, presumably globulin, which readily coagulates in water.

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Metabolism of Trichloroethylene in the Bovine.* (22019)

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It has been suggested(1) that the aplastic anemia which is observed in the bovine(2) fed certain specimens of trichloroethylene-extracted soybean oil meal is induced by residual trichloroethylene in the toxic meal. Prolonged feeding of relatively large amounts of trichloroethylene however failed to produce harmful effects in calves(3). This suggests that the bovine, like some other species which are relatively resistant to the toxic effects of trichloroethylene-extracted soybean oil meal (4) can metabolize trichloroethylene. In fact, urochloralic acid has recently been isolated from the urine of calves fed trichloroethylene and identified through degradation and synthesis of a derivative as 2′, 2′, 2′-trichloroethyl-β-D-glucopyranosiduronic acid(5). It was thus established that the bovine, like the dog(6), can metabolize trichloroethylene to trichloroethanol and excrete the latter as a conjugate. It remained to be determined however, to what extent this occurs and if trichloroacetic acid, a major metabolite of trichloroethylene in mice(7), rats(8), dogs (9) and man(10) is also produced by the bovine.

Methods. Four female Holstein calves weighing from 100 to 112 lb were placed into individual metabolism cages, fed 5 lb of whole cows' milk twice daily and given water *ad libitum.* Trichloroethylene was administered orally in gelatin capsules twice daily with the milk in the following total daily amounts: Calf A, 3 g for 5 days; calf B, 12 g for 5 days; calves C and D, 12 g for 4 days. The urine was collected in brown bottles under toluene. Separate 24-hour specimens were obtained before, during and after the period of administration of trichloroethylene. Aliquots of the urine were used for determination of trichloroacetic acid, "total trichloro compounds" and trichloroethylene; the latter was also determined in the toluene layer. The analytical methods used have been described recently(11). Briefly they consist in the quantitative application of the Fujiwara alkali-pyridine reaction. In our modification trichloroacetic acid, trichloroacetaldehyde, chloroform and trichloroethanol do not interfere with the determination of trichloroethylene. The latter as well as chloroform can be removed from the urine prior to the determination of trichloroacetic acid or of "total trichloro compounds." Trichloroethanol and urochloralic acid do not interfere with the determination of trichloroacetic acid but all 3 compounds are included in the determination of "total trichloro compounds." The difference between "total trichloro compounds" and trichloroacetic acid (calculated on a molar basis as trichloroethanol) corresponds to free and combined "trichloroethanol" including urochloralic acid. Other products of metabolism of trichloroethylene which no doubt exist have not been definitely recognized. If, after oxidation they would give a positive Fujiwara reaction they would be included as "total trichloro compounds" and would thus contribute

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TABLE I. Trichloroacetic Acid and "Trichloroethanol"* in Urine.

24 hr interval	Calf A (3 g/day) TCA†	TCE‡	Calf B (12 g/day) TCA†	TCE‡	Calf C (12 g/day) TCA†	TCE‡	Calf D (12 g/day) TCA†	TCE‡
	mg							
1	11.8	500	16.2	1169	0	793	0	911
2	18.6	596	43.1	1339	7.3	613	14.6	692
3	33.6	848	109	2487	15.8	1453	37.4	2116
4	48.3	1475	78.0	1346	37.2	2350	59.3	2595
5	46.6	576	145	1973	79.3	1651	97.9	1697
§ 6	36.5	75	121	116	20.7	39.5	28.2	53
7	25.0	8.6	73.5	16.4				
8	16.9	0	37.2	5.5				
9	16.6	0	38.1	0				
10	11.7	0	16.8	0				

* Includes urochloralic acid and other compounds which give a positive Fujiwara reaction after oxidation.

† Trichloroacetic acid.

‡ "Trichloroethanol" corrected for trichloroacetic acid excreted.

§ Daily dose of trichloroethylene given in parentheses below letter of each animal; fed on days above horizontal line.

to the fraction which, with this reservation in mind, is referred to as "trichloroethanol."

Results. No ill effects on the calves were observed as a result of the administration of trichloroethylene. No compounds giving a positive Fujiwara test were detected in the urine collected before the administration of trichloroethylene.

Trichloroethylene could not be detected in the aqueous phase of the collected specimens even after extensive aeration and collection of the volatile components in toluene. However, while trichloroethylene was fed, the toluene layer used to preserve the urine contained from 3 to 6 micromoles of trichloroethylene per day which in the case of calves B to D is less than 0.01% of the dose fed.

The presence of trichloroacetaldehyde, an assumed product of metabolism of trichloroethylene(6) and the probable direct precursor of trichloroacetic acid and trichloroethanol could not be detected by a test(11) which is sensitive to at least 15 μg; lack of evidence for the presence of trichloroacetaldehyde is in accord with the observations of Butler with dogs(6).

Table I summarizes the data obtained with determinations of trichloroacetic acid and "trichloroethanol" in the urine. Although trichloroacetic acid was not identified as such in the urine it was detected through its behavior in the Fujiwara reaction(11). During the administration of trichloroethylene, the concentration of trichloroacetic acid in the urine increased gradually; it decreased again gradually after feeding of trichloroethylene had ceased. The molar recovery of trichloroacetic acid in the urine of calves A and B was only 1.4 and 1% respectively of the total amount of trichloroethylene administered and only 5.6 and 7.2% respectively of the "total trichloro compounds" found in the urine. Continued excretion of trichloroacetic acid after exposure to trichloroethylene has also been observed in humans and in dogs(6). However, in those species the proportion of trichloroethylene metabolized to trichloroacetic acid appears to be much higher than in the bovine. Butler estimated that in dogs (6) trichloroacetic acid was excreted to the extent of about ⅓ to ½ of the amount of trichloroethanol. In the urine of rats(8,12) however, exposed to 80 mg of trichloroethylene per liter of air, the concentration of trichloroacetic acid was only 1.2 mg per liter. Ahlmark and Forssman(8) found that man excreted from 6 to 18% of inhaled trichloroethylene as trichloroacetic acid in the urine. The highest concentration of trichloroacetic acid (50 mg per liter) was found in calf B on the fifth day. In man, Grandjean *et al.* (13) have found that a mean of 13% of the trichloroethylene inhaled during a working day was excreted as trichloroacetic acid; the concentration of trichloroacetic acid in the urine of their subjects ranged from 8 to 440 mg per liter with a mean of 84.7 mg.

"Trichloroethanol" was excreted by the calves rapidly and extensively after administration of trichloroethylene. The largest amount, 2.6 g was found in the urine of calf D on the fourth day after feeding trichloroethylene; the concentration was 940 mg per liter in this specimen of urine. When feeding of trichloroethylene was discontinued "trichloroethanol" disappeared from the urine within 2 to 3 days. Feeding of trichloroethylene at the higher level increased the excretion of "trichloroethanol" but not proportionately. The recovery of "total trichloro compounds" in the urine during the whole period was only 25.4, 13.3, 13.5 and 15.2% respectively for calves A through D, calculated on a molar basis. Our analytical procedure did not permit differentiation between free and combined trichloroethanol. Butler (6) has reported that dogs excreted from 42 to 66% of trichloroethanol which was injected intramuscularly, mostly in conjugated form.

Discussion. We are not aware of experiments in which the metabolism of trichloroethylene was studied after oral administration; for this reason and because the bovine has not previously been used for studies of the metabolism of trichloroethylene comparisons with other species are somewhat tenuous. However, these experiments show that some of the products of metabolism of trichloroethylene in the bovine are similar to those observed in other species. While it is evident that the calf can "detoxify" trichloroethylene to a considerable extent and excrete urochloralic acid as well as trichloroacetic acid a major fraction of the trichloroethylene administered remained unaccounted for. It is possible that this portion may have been in part exhaled or excreted with the feces, or metabolized to monochloroacetic acid(14) or to inorganic chloride(15) and thus would have escaped detection by the Fujiwara test.

Summary. Following the oral administration of 3 to 12 g of trichloroethylene to calves weighing about 100 lb, about 1% of this compound was excreted in the urine as trichloroacetic acid and from 13 to 25% as trichloroethanol (free and combined). Only traces of trichloroethylene were excreted in the urine. No trichloroacetaldehyde could be demonstrated in the urine with the method used. Some of the metabolic products of trichloroethylene in the bovine are the same as those produced by man and dogs.

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Eosinophiles in Passive Anaphylaxis.* (22020)

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It has long been recognized that blood and tissue eosinophilia is a common accompaniment of antigen-antibody activity(1,2,3). The mechanism responsible for the eosinophilia, however, has not been clear. To determine whether a state of active antibody production is a prerequisite for its development the following experiments on passive anaphylaxis were undertaken.

Materials and Methods. Three times crystallized egg albumin (Ea) was used as antigen. The antiserum was from a rabbit ($R194_{4+5}$, ref. 4), which had been injected with a total dose of 40 mg of antigen over a 2 months' period. This antiserum had a precipitable anti-Ea N content at the time of use of 308 μg per ml. Animals for sensitization and for controls were 250 to 325 g guinea pigs. Injections were made into leg veins. Sensitization was accomplished with 23 μg antibody N given intravenously 48 hours prior to the shocking dose of antigen (6 μg Ea N). Blood counts were done on peripheral blood, use being made of ear, leg, and foot veins for this purpose. To prevent coagulation of the blood, it was necessary to wipe the cleaned skin surface and puncture needle (#23 hypodermic needle) with a piece of cotton previously dampened with heparin. White cell counts were performed in a Spencer bright-line counting chamber. Bloods for the total leukocyte counts were diluted 20-fold in a white cell pipette with a solution of acetic acid and methylene blue. Eosinophile counts were done on 10-fold diluted blood in a freshly prepared solution of Phloxin in 0.1% propylene glycol. Both sides of the counting chamber were filled in each instance, four large squares on each side being used in enumerating the total white count and the entire ruled area being used in enumerating the absolute eosinophile count.

TABLE I. Absolute Eosinophile Counts after 6 μg Egg Albumin Nitrogen Intravenously in 9 Sensitized and 7 Non-Sensitized Guinea Pigs.

	Time in hr							
	0	¼–½	1	2	4	6	12	24
	Sensitized with 23 μg anti Ea N							
	201		195	294	165	243		
	33	51	198	165		357		
	66	171		120	141	207		
	30		81	360	240	204		204
	33		6	255	90	123		162
	33	15	99	381	174	147	78	
	12	87	315	135	105	126		
	27	21	117	99	33	21		
	24	57	111	174	288	21		
Mean ± S.E.	51 ± 19	67 ± 24	140 ± 34	220 ± 35	155 ± 29	161 ± 36		
	Non-sensitized controls							
	18	3	24	18	36	21		9
	36	30	27	36	45	36		9
	30	21		30	36	39		30
	30	6	174	36	42	72	27	12
	45	15	66	15	21	9	18	9
	30	15	15	27	18	21		
	3	9	18	24	12	24		
Mean ± S.E.	27 ± 5.1	14 ± 3.4	54 ± 25	29 ± 3.0	30 ± 4.9	32 ± 7.7		
P	>.2	.04	.08	<.01	<.01	<.01		

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* Supported by a grant from the A. D. Williams Fund of the Medical College of Virginia.

† A. D. Williams Postdoctoral Fellow.

TABLE II. Total Leukocyte Counts after 6 μg Egg Albumin Nitrogen Intravenously in 9 Sensitized and 7 Non-Sensitized Guinea Pigs.

	Time in hr							
	0	¼–½	1	2	4	6	12	24
	Animals sensitized with 23 μg anti Ea N							
	9400		9150	13400	9400	9020		
	2150	8400	10800	11100		7600		
		18850		9050	9050	9400		
	6100			14400	6200	7900		8800
	9800			9750	5350	10400		11900
	9750	12200	15750	7950	3100	9650	9250	
	9950	11300	11050	10950	8400	6600		
	14400	9550	8300	12400	4650	5050		
	7150	8500	6850	9800	11100	11300		
Avg	8600	11500	10300	11000	7160	8550		
	Non-sensitized controls							
	5200	10200	8000	11300	8600	16200		5750
	12900	13100	9500	10100	10000	13700		11400
	4900	4450		8900	7200	9150		6300
	6150	5200	6700	5250	6000	3950	7600	5900
	16200	8850	8100	9750	5900	5150	10250	11750
	22850	11200	16450	22050	21000	21850		
	7550	4600	15450	5600	850	4600		
Avg	10550	8230	10600	10400	8510	10700		

Results. Table I and Fig. 1 present the eosinophile counts and Table II presents the total white cell counts in the animals studied. It is clear that there was a significant rise of the eosinophiles in sensitized animals injected with 6 μg of Ea N. This eosinophilia occurred independently of any corresponding rise in the total number of white cells and was maintained for 24 hr in 2 animals examined at this interval. In spite of some variability in the serial counts in these animals, probably due to technical difficulties, the eosinophile differences observed at 2, 4 and 6 hours were statistically highly significant.

It was of interest to note in several instances in a preliminary part of this study moderate eosinophile responses in normal, non-sensitized animals after large (160 μg) doses of Ea N (Table III). This effect was not seen in normal animals at the smaller doses of Ea used later in the anaphylaxis experiments (Table I).

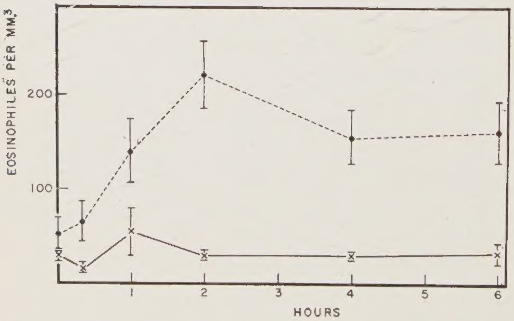

FIG. 1. Mean values and stand. errors of eosinophile responses of sensitized (– – – –) and normal (———) guinea pigs to 6 μg Ea N.

Discussion. That eosinophilia may occur in anaphylaxis independently of active antibody formation is established.‡ This suggests that the stimulus for the eosinophilia is to be found in a product released by the antigen-antibody reaction, or in one of the enzymes activated by the reaction(6). Particularly suggested is histamine itself, which has been reported to produce an eosinophilia in the guinea pig(2,7).

The finding of an eosinophilia in unsensitized guinea pigs after the larger Ea dose recalls the reports by Vaughn(7,8) that eosinophilia may be produced in normal guinea pigs by the injection of ascaris polysaccharide. However, the proper interpreta-

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‡ After the conclusion of this study, we became aware of a comment by Campbell (Discussion, reference 5) that he had observed eosinophilia in passive, as well as in active anaphylaxis. The present paper, therefore, confirms and extends his earlier finding.

TABLE III. Blood Eosinophile Response to Injection of Large (160 μg) Doses of Egg Albumin Nitrogen in 4 Normal Guinea Pigs.

	Time in hr					
Route	0	¼–½	1	2	5	6
I.V.	56	53		175	175	259
”	118	109		68	156	237
I.P.	188	255	350	693	394	160
S.C.	55	33	49	222	316	38

tion of this finding is not clear. The claim (8) that the polysaccharide, independent of immune reaction to it, is primarily toxic and a stimulus to histamine release is not fully supported by the evidence, though this interpretation is certainly possible. Numerous instances have been described both in man and in the experimental animal, wherein antibody to various antigens may be "naturally" acquired. In man, well-documented instances would include the "natural" isoagglutinins and "normal" antidextrans(9). It would be difficult to prove that such "natural" antibodies did not play a role in the above instances. Further, since relatively large doses of antigen have been required for the production of eosinophilia in normal animals the possibility exists that such antibodies might be antibodies to antigen impurity(4). Therefore the experiments in which eosinophilia has been observed in normal animals may need reinterpretation, and particularly is this indicated in those cases(8) in which the animals were noted to die in convulsions, or with dyspnea, after the eosinophilia-producing injections. Such symptoms were not seen in the animals reported in Table III.

Summary and conclusions. It has been shown that eosinophilia in anaphylaxis is not dependent upon active antibody formation by the shocked animal. The development in several instances of a mild eosinophilia following the injection of relatively large doses of Ea into normal guinea pigs was noted.

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Role of Hyperkalemia in Production of Ventricular Fibrillation Following Hypercapnia.* (22021)

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Rapid return to air breathing following 2 to 4-hour periods of high CO_2 breathing regularly produces hypotension and cardiac arrhythmias in dogs, and may produce ventricular fibrillation and death(1). Recently Sealy, Young, and Harris(2,3) have confirmed these results and have extended them in an attempt to determine the cause of the severe cardiac effect of this rapid fall in CO_2 tension and hydrogen ion concentration. They were impressed, as we were, with the similarity between the electrocardiograms recorded in the immediate post-hypercapnic period and those characteristic of hyperkalemia. These inves-

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tigators found a rise in serum potassium during hypercapnia which they believe is causally related to cardiac response.

Experiments reported in this paper suggest that the elevated plasma potassium is not sufficient of itself to produce the severe cardiac effects, but that a rapid decrease in arterial blood CO_2 tension and hydrogen ion concentration in the presence of elevated sub-lethal plasma potassium concentrations may produce cardiac arrest or ventricular fibrillation.

Methods. Five groups of experiments were carried out on dogs anesthetized with either sodium pentobarbital (Group 3) or sodium Pentothal (Groups 1, 2, 4, 5) administered intravenously. Blood pressures were recorded from a femoral artery by means of a strain gauge and one channel of a 2-channel direct writing oscillograph. Electrocardiograms were recorded on the second channel of the oscillograph using limb leads. A catheter for obtaining blood samples was passed through the femoral artery into the aorta and tied in place. Gas mixtures were administered through either an endotracheal tube or a tracheotomy tube from an open system.

In experiments in which KCl solution was administered, it was infused as a .16 M solution in an intravenous drip at approximately 100 drops per minute. Potassium determinations were carried out on a protein free filtrate of plasma with a flame photometer. Syringes for drawing 6 ml blood samples were prepared by allowing 0.1 ml of heparin solution containing 1 mg of heparin to dry overnight in each syringe in a warm oven.

Group 1. Thirteen dogs breathed 30% CO_2-70% O_2 for 2 hr followed by 40% CO_2-60% O_2 for an additional 2 hr, and were then returned to air breathing. Blood samples for assay of plasma potassium concentrations were drawn before starting the CO_2 breathing, at intervals during the 4 hours of CO_2 inhalation, and at intervals during the first hour after returning the animals to air breathing.

Group 2. Eleven dogs were administered KCl solution, while breathing air, until cardiac arrest or ventricular fibrillation appeared. Blood samples were drawn and electrocardiograms and blood pressures recorded before starting the KCl infusion and at intervals during infusion.

Group 3. Control blood samples and recordings were obtained on 8 animals who were then given 30% CO_2 in oxygen to breathe. After 10 minutes on this mixture an intravenous drip of KCl solution was started with the dogs continuing to breathe 30% CO_2. This was continued until ventricular fibrillation or cardiac arrest appeared.

Group 4. Eight dogs were given 30% CO_2 for 2 hours followed by 40% CO_2 for 2 hours and then while they continued to breathe 40% CO_2 an intravenous drip of KCl was started and continued until the heart stopped or the ventricles fibrillated.

Group 5. Thirty % CO_2 for 15 minutes followed by 40% CO_2 for 10 minutes was administered to 6 dogs. While the animals continued to breathe 40% CO_2, KCl solution was given as an intravenous drip until plasma potassium concentration was thought to be about 8 to 9 mEq/L. as judged by the amount of KCl given and by the electrocardiograms. This usually required 5 to 8 minutes. At this point the dog was changed from the high CO_2 mixture to 100% oxygen and hyperventilated at a rate of 50 breaths per minute and a positive pressure of 20 cm of water by means of a positive pressure respirator.

In all experiments during administration of KCl solution, blood pressures and electrocardiograms were recorded at intervals of one

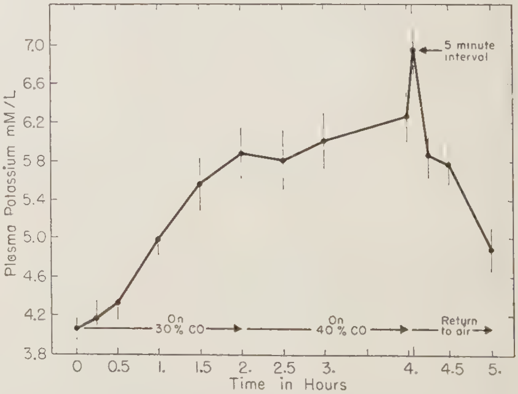

FIG. 1. Mean plasma potassium concentrations in 13 dogs before, during and following 4 hr of breathing 30% and 40% CO_2 in oxygen. Vertical bars extend one stand. error on each side of the mean.

TABLE I. Influence of Inhalation of High CO_2 Concentrations on Terminal Plasma Potassium Concentrations.

Group		No. of dogs	Plasma potassium conc., mEq/L Control	Before drip	Final
2	Breathing air	11	3.62 ± .132*		14.39 ± .329
3	Breathing 30% CO_2 for 15 min.	8	3.39 ± .090	3.66 ± .131	12.98 ± .572
4	Breathing 30% CO_2 for 2 hr followed by 40% CO_2 for 2 hr	8	3.84 ± .241	7.10 ± .416	13.79 ± .890

* Stand. error of mean.

to 2 minutes until severe cardiac arrhythmias appeared, and then continuously until cardiac arrest or fibrillation occurred. Blood samples were drawn at 5 minute intervals during the first few minutes of KCl infusion and at shorter intervals during the remainder of the experiment. An attempt was made to draw the final sample just before cardiac arrest took place. By watching the electrocardiogram and blood pressure tracing this was usually accomplished successfully.

Results. Fig. 1 presents the plasma potassium concentrations of 13 dogs in Group 1 during and following 4 hours of hypercapnia. Two of these dogs died in ventricular fibrillation after 5 minutes of air breathing and are not represented in the data after that time. A gradual increase in plasma potassium concentration during the hypercapnia was a regular finding. In 12 of 13 cases a further and more rapid increase took place during the first 5 minutes of air breathing. Immediately thereafter the concentration began to decrease but was still somewhat elevated one hour after the dogs had been returned to air breathing. The highest value recorded in any of these animals was 8.2 mM/L. at 5 minutes of air breathing following the 4 hours of hypercapnia. This dog died in ventricular fibrillation within seconds after the blood sample was drawn.

To be sure the peak concentration was not being missed, blood samples were drawn at one or 2-minute intervals for 12 minutes following hypercapnia in 4 dogs. In every case the highest concentration was found between 4 and 6 minutes. Table I presents the data for Groups 2, 3, and 4. The mean plasma potassium concentration at cardiac arrest in the dogs breathing air (Group 2) was 14.39 mM/L. This value agrees well with those reported by others(4). When the dogs were made hypercapnic prior to starting the KCl drip (Group 3) cardiac arrest occurred when the plasma potassium concentration averaged 12.98 mM/L. In the 8 dogs that breathed high CO_2 mixtures 4 hours before the KCl infusion began, the average plasma potassium concentration at cardiac arrest was 13.79 mM/L. Although hypercapnia of this degree appeared to lower the plasma potassium concentration at which cardiac arrest appeared, the differences were not impressive and in no instance in any of these groups did arrest or ventricular fibrillation occur when the plasma potassium level was as low as 8.2 mM/L., the highest level found to occur spontaneously during the rapid fall in pCO_2 and hydrogen ion concentration following 4 hours of hypercapnia. In other words no overlap in the terminal plasma potassium values between Group 1 and Groups 2, 3, and 4 appeared.

The possibility still existed, however, that the hyperkalemia and a rapid rise in pH and/or fall in CO_2 tension might be the necessary conditions to produce cardiac arrest or ventricular fibrillation. In earlier work we had demonstrated many times that blood pH falls to approximately 6.7 when a dog breathes 40% CO_2 for 15 minutes to 30 minutes and that rapid reversal of this hyercapnic, acidotic state is not followed by cardiac arrest or fibrillation as it commonly is when the hypercapnia has lasted 2 or more hours. Thus it appeared that the rapid fall in CO_2 tension or rise in pH alone were not producing the untoward cardiac effects. The present data indicate that the plasma potassium level attained following 4 hours of hypercapnia was not sufficient of itself to produce this effect.

To determine whether the combination of a rapid fall in hydrogen ion concentration and pCO_2 in the presence of a sub-lethal level of plasma potassium would produce cardiac ar-

TABLE II. Effect of Rapid Reduction in pCO_2 in Presence of Elevated Plasma Potassium Concentration.

Dog No.	Plasma potassium concentrations, mM/L Control	High CO_2, 25 min.	KCl drip, 5 min.	3 to 7 min. on O_2	Result
1	4.2	4.6	10.2	9.3	Ventricular fibrillation
2	3.9	3.9	8.7	8.4	" "
3	3.7	3.8	8.4	11.5	" "
4	3.6	4.0		6.3	Cardiac arrhythmia (dog lived)
5	4.2		10.0	10.0	Ventricular fibrillation
6	3.6		11.9	11.3	Cardiac arrest

rest or fibrillation, the experiments in Group 5 were carried out. The data for this group of experiments are given in Table II. In one dog the plasma potassium concentration during the hyperventilation was only 6.3 mM/L. The electrocardiogram on this dog showed cardiac arrhythmias but the dog did not die. In 3 other cases the potassium level when fibrillation began was high enough that the hyperkalemia alone might have produced the result, although the values were well below the average for the dogs that received KCl during the hypercapnia. In 2 dogs of this series ventricular fibrillation occurred during the first 7 minutes of hyperventilation at a time when the plasma potassium concentrations were 8.4 mM/L. and 9.3 mM/L. respectively.

Comment. The pattern of changes in plasma potassium concentration during inhalation of high CO_2 mixtures appears to be different in dogs from that which has been observed in cats. Cattell and Civin(5) in attempting to separate the hypoxic effect from the hypercapnia effect of asphyxia, administered 10% CO_2 to cats and found a marked but temporary rise in serum potassium. MacKay(6) followed up this work by administering 35% CO_2 to cats and obtained similar results. An increase amounting to 3 to 5 times the normal value appeared within 10 minutes after beginning the CO_2 administration and returned to normal in 30 minutes. In addition a second rise took place when the cats were returned to air breathing. The first of these increases but not the second was blocked by tying off the blood supply of the abdominal viscera.

In dogs there appears to be a gradual increase in plasma potassium level during 4 hours of high CO_2 breathing, with an increase in slope during the first 5 minutes of air breathing following the CO_2. This latter change is similar to that reported in cats. In order to be sure that very rapid changes were not appearing during the first few minutes of CO_2 breathing in the dog, blood samples were drawn at 5-minute intervals for the first 30 minutes in several animals. All of the values obtained fell between the control and 30-minute sample. The explanation for this difference between cats and dogs in mobilization of potassium is not available at present.

Young, Sealy, and Harris(3) found a simultaneous rise in cardiac muscle potassium and plasma potassium concentration in the rat following 2 to 4-hour periods of exposure to 35 to 45% CO_2. What changes occur in heart muscle potassium during the rapid shift in blood pH are not known. The work of Fenn and Cobb(7) would suggest that heart muscle potassium should fall at the time carbon dioxide tension is falling. They found a shift of potassium from frog muscle to blood when carbon dioxide tension was reduced. Young *et al.*(3), however, found the cardiac muscle potassium still elevated after rats were removed from an atmosphere of high CO_2 to air. It may be that changes in some other electrolyte are playing a role in the cardiac effect. We have found blood pH regularly falling to approximately 6.7 when the animal breathes 40% CO_2. On changing to air the pH rises above 7.2 in 5 minutes or less. It is possible that this rapid shift in hydrogen ion concentration itself is responsible for the altered response of the heart.

A post-hypercapnic phenomenon similar to that exhibited by dogs has been demonstrated in man(8,9) and it has been suggested that certain unexplained post-surgical deaths may be due to a rapid reduction in CO_2 tension which has become elevated during anesthesia. It would be interesting to know what changes take place in plasma potassium concentration in the human during prolonged exposure to severe hypercapnia. It seems likely that it is elevated but it would be important to know how rapidly and to what extent, as a function of time and CO_2 concentration, since this would have a bearing on the susceptibility of the heart to fibrillation or arrest when the CO_2 tension is reduced rapidly.

Summary and conclusions. 1. Dogs regularly show an increase in plasma potassium concentration during breathing of 30% and 40% CO_2 mixtures. Immediately following return to air breathing, there is a further sharp rise in this concentration. This secondary rise reaches a peak after 5 minutes of air breathing. 2. Concentration of plasma potassium following 4 hrs of high CO_2 breathing is not as high as that which is necessary to produce fibrillation or arrest when KCl solution is administered as an intravenous drip to normal or acidotic dogs. 3. Rapid return to air breathing following 30 minutes of high CO_2 breathing does not produce cardiac arrhythmias, ventricular fibrillation, or cardiac arrest in dogs. However, when this rapid elevation in blood pH and/or fall in pCO_2 takes place in the presence of sub-lethal but elevated plasma potassium concentrations, cardiac arrest or ventricular fibrillation may occur. Although this does not provide an explanation of the mechanism involved, it appears that the combination of elevated but sub-lethal plasma potassium concentration and a rapid fall in CO_2 tension and/or hydrogen ion concentration, work together to produce the damage to the heart.

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Photometric Method for Estimation of Elastase Activity.* (22022)

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The presence of an enzyme in animal pancreatic extracts which solubilizes elastin was first described by Balo and Banga(1). The activity was measured gravimetrically and by Kjeldahl nitrogen. Using the same technics, Hall(2), and later Lansing *et al.*(3), confirmed the presence of this enzyme in extracts of hog pancreas. Since both methods are somewhat cumbersome, a simpler colorimetric procedure was devised; it permits the direct measurement of the amount of dyed elastin which goes into solution when the dyed substrate reacts with pancreatic extracts.

Material and methods. Substrate. Purified elastin was prepared by the Lansing, Alex and Rosenthal(4) modification of the method of Lowry *et al.*(5). The ligamentum nuchae of horses was freed of the thin investing fascia and then ground into small pieces in a meat

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grinder. This well-ground material was covered with methanol and refluxed over a hot water bath for one hour. The alcohol was decanted, the residue covered with ether and refluxed for another hour. The residue was allowed to dry, added to 0.1 N sodium hydroxide which had been preheated to 93-98°C and the mixture kept at this temperature for 45 minutes. The sodium hydroxide was decanted and the residue washed thoroughly with water. This elastin (20 g) was air-dried, ground to 40-mesh size in a Wiley mill, and suspended in an orcein solution composed of 1 g of orcein in 100 ml of 70% ethanol to which 1 ml of concentrated hydrochloric acid had been added. The stained elastin was then washed repeatedly with large volumes of 70% ethanol and distilled water until both the ethanol and the aqueous washings were color-free. The stained elastin was finally washed with 70% ethanol and the air dried residue ground in a mortar. Samples of this substrate incubated in an enzyme-free system, as described in the Procedure, gave no color change in the supernatant. Quadruplicate nitrogen determinations of this elastin preparation by the Conway micro-diffusion method(6) gave an average of 14.5%.

Buffers: Tris (hydroxymethyl) aminomethane buffer 0.2M, pH 8.8, prepared according to Gomori(7). This buffer will hereafter be referred to as Tris. Phosphate buffer 0.7M, pH 6.0, prepared according to Sörenson (8).

Enzyme Preparation: This was prepared by extracting 10 g Viobin† (defatted autolyzed pork pancreas) with 50 ml N/40 hydrochloric acid for 20 minutes on a magnetic stirrer at room temperature and centrifuging the preparation at 12,000 rpm in an International Model PR-1 high-speed refrigerated centrifuge. The residue was reextracted with another 50 ml N/40 hydrochloric acid for 20 minutes and recentrifuged. The pooled clear supernatant measured approximately 90 ml. This crude extract had a pH of about 5, and was adjusted to pH 8.8 with 1N sodium hydroxide before use. There was no appreciable loss of elastolytic activity by keeping this preparation in the refrigerator for several weeks. Using our assay procedure, 10 g of Viobin extracted with 100 ml N/40 hydrochloric acid yielded a total of 12,000 to 13,000 elastase units when one elastase unit is defined as the amount of enzyme activity required to solubilize 1 mg of elastin substrate in 20 minutes at 37°C under our experimental conditions.

Procedure. Twenty mg portions of the dyed substrate were placed in small test tubes (13x125 mm) and 1 ml of Tris buffer, pH 8.8, 1 ml water, and 1 ml of suitably diluted enzyme preparation adjusted to pH 8.8 added to each. The tubes were then stoppered, placed in the horizontal position in a mechanical shaking device, which was immersed in a constant temperature water bath at 37°C. The shaker moves at a uniform rate of 46 excursions per minute. At the end of 20 minutes the tubes were removed from the bath and 2 ml of 0.7M phosphate buffer, pH 6, added to each tube to stop the enzyme reaction. With this drop in pH the color of the reaction mixture changed from a bluish purple to a reddish purple. This mixture was then centrifuged and the per cent transmission of the supernatant read on a Model DU Beckman Spectrophotometer at 590 mu. The results were then converted into specific activity, *i.e.*, elastase units per milligram enzyme preparation used.

Standard curve: Duplicate portions of the substrate, ranging from 5 mg to 25 mg, were placed in the test tubes. To each tube 1 ml of 0.2M Tris buffer, 1 ml water and 1 ml of enzyme preparation were added and the mixture incubated as described until all the elastin went into solution. This process took from 24 to 48 hours, depending on the strength of the enzyme preparation used. Then 2 ml of the phosphate buffer and 5 ml of water were added, giving a final dilution of 1:10. In all tubes a very small amount of insoluble material remained and this was attributed to non-elastin contamination of the substrate. This insoluble residue was estimated by nitrogen determination and gave an average of 3.8%.

Results. Following centrifugation the du-

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† Viobin was generously contributed by the Viobin Corp., Monticello, Ill.

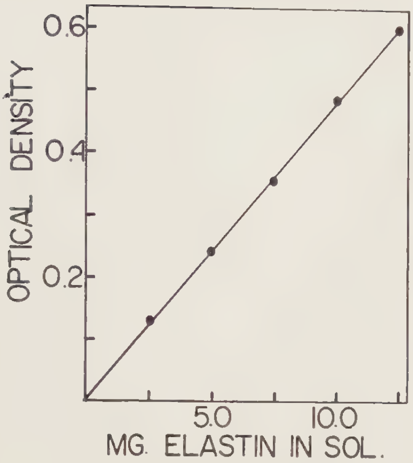

FIG. 1. Relationship between mg elastin dissolved and optical density (590 mu) of the orcein dye liberated.

plicate samples were read at 590 mu on the Beckman Spectrophotometer and the readings plotted as optical density vs mg elastin per 5 ml solution (Fig. 1). The duplicate determinations were in good agreement. Regardless of the analytical procedure, it would seem that standardization of a new elastin preparation would always be indicated, since there may be slight variation in the constituents of any given batch, and there is no reason to assume that any of these preparations represents pure elastin.

Time curve. By using a stop watch, tubes containing the substrate, buffer, water and enzyme mixture, as described, were withdrawn from the water bath at intervals and the reaction stopped by the addition of the phosphate buffer. Results were recorded as time in minutes vs milligrams elastin in solution (Fig. 2).

Dilution curve. Dilutions of a lyophilized enzyme preparation, varying from 0.2 to 5.0 mg elastase powder per ml, were used, with an incubation time of 20 minutes. The results were plotted as milligrams dissolved elastin vs enzyme concentration, and they followed a zero order reaction when less than 7 mg or 35% of the 20 mg substrate used was solubilized (Fig. 2).

pH Optimum. Hall and Gardiner(9) showed that the pH optimum of elastolytic activity lies between 8.7 and 9.2, with a different peak for substrates of different animal origin. On horse ligamentum nuchae, prepared as described, the pH optimum for our enzyme preparation lies between 8.7 and 9.4. The inadequacy of the 0.1N carbonate buffer, pH 10.23, used by Balo and Banga, was verified by us, using the colorimetric method. The pH after incubation dropped to levels varying from 9.8 to 8.9. Buffers of sufficient molarity to hold the pH of the reaction mixture at 10.23 gave minimal elastolytic activity.

Comparison of methods. Different amounts of the elastin substrate were weighed on an analytical balance and exposed to the enzyme buffer mixture until varying amounts of the elastin had gone into solution. The supernatant was removed after centrifugation and the amount of solubilized elastin determined by the colorimetric method described. The nitrogen content was assayed by the Conway micro-diffusion method, using 1 ml of the supernatant. Appropriate controls consisting of identical mixtures of the enzyme buffer solution were also assayed for their nitrogen content. For the gravimetric procedure the residues were oven-dried at 100°C and weighed on an analytical balance, the amount of elastin in solution being the difference between the original weights and that of the residues. The results of all three methods are recorded in Table I.

Discussion. The assay procedure described

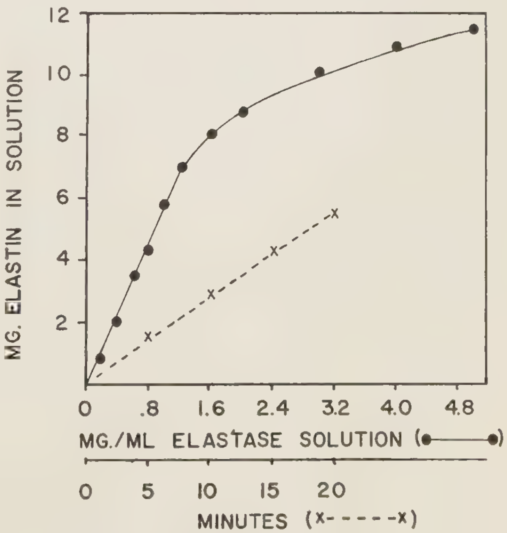

FIG. 2. Effect of time and of elastase concentration on dissolution of elastin.

TABLE I. Milligrams Elastin Dissolved by Action of Elastase as Measured by:

Gravimetric procedure	Colorimetric method	Total nitrogen in solution
21.8	21.8	21.3
21.3	22.7	25.6
19.7	20.2	19.3
17.9	18.2	17.8
18.0	17.5	17.6
10.1	10.9	11.1
8.9	7.9	8.2
12.5	12.5	12.7
11.0	11.6	11.1
4.5	3.7	4.8
3.0	2.9	2.4
5.1	4.3	5.9
18.8		17.8
18.0		19.1
26.8		26.0
33.0		33.1
	19.9	19.5
	3.4	2.4
	11.2	12.7
17.9		17.3
11.8		11.0
19.7		18.5

here has the advantage of rapidity, simplicity and reproducibility, as well as the production of a visible reaction, so that one gauges at a glance whether activity is present and to what degree, without first having to complete the procedure. Hall(10) assayed the enzyme preparation by incubation for 18 hours, without agitation. It would appear that one cannot determine with accuracy the specific activity of the enzyme preparation under conditions where the dissolution of elastin is no longer linear. The risk of bacterial contamination is reduced by using the shorter incubation period in our method. No inactivation of the enzyme was noted by the amount of agitation used by us; instead, it was found that the enzymatic activity was increased up to 100% as compared to incubation without agitation.

When the same enzyme solution is tested against batches of horse ligamentum nuchae substrate prepared at different times, the activity changes. This is most probably a result of variation in particle size and in the amount of dye absorbed by the elastin. This variable may be cited as a disadvantgae of the method, but standardization of the substrate each time a new batch is prepared would appear to be necessary, irrespective of the analytical procedure used. The problem can be minimized by preparing larger quantities of the substrate each time.

Summary. A colorimetric method for the estimation of elastase activity has been presented. When dyed elastin substrate prepared from horse ligamentum nuchae is used, the pH optimum lies between 8.7 and 9.4, and the reaction velocity is linear only when less than 35% of the elastin is dissolved. When compared with the gravimetric and nitrogen procedures, this method has the advantages of rapidity and simplicity.

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Comparative Effects of Anesthetic Agents on Incidence of Ventricular Fibrillation During Hypothermia. (22023)

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Cardiac ventricular fibrillation and asystole are two factors which have produced a limit for experimental and clinical hypothermic procedures. Fibrillation has generally occurred at higher temperatures than asystole. Elimination of factors which tend to cause fibrillation might therefore permit the use of lower temperatures for such procedures. Choice of anesthetic agent is conceivably involved in the production of fibrillation. Available data are on the whole inconclusive as to the role that various anesthetic agents play, although Covino *et al.*(1) state that ventricular fibrillation occurs less frequently in dogs receiving thiopental or ether than in those receiving pentobarbital.

It therefore seemed desirable to study the effects of commonly used agents, ether, cyclopropane and pentobarbital with regard to their effect on production of ventricular fibrillation or asystole during hypothermic anesthesia.

Methods. Healthy mongrel dogs of both sexes ranging in age from 6 to 12 months and weighing between 3.6 and 9.1 kg were used. No premedication was given. Eleven dogs were anesthetized with cyclopropane and 10 anesthetized with ether. They were intubated as soon as depth of anesthesia was sufficient to permit it. They were connected to a closed to-and-fro CO_2 absorption system with controlled hyperventilation at 50 ± 5 respirations per minute. The hair was removed from body, neck and proximal parts of legs. A rectal thermometer was inserted at least 10 cm. Needle electrodes for Lead II of electrocardiograph were attached. The animals were immersed on their sides in ice bath at 1 to 3°C with only head and part of neck elevated above bath. The level of anesthesia was maintained so lateral canthus reflex was lost and medial canthus reflex present. This provided anesthesia which would be adequate for surgical procedures. When rectal temperature reached 22°C artificial respiration with 100% oxygen was continued with a controlled phase respirator regulated at 50 ± 5 respirations per minute. A third series of dogs was given 30 mg per kilo of pentobarbital intravenously and intubated as soon as possible. Hair was removed, rectal thermometer inserted, needle electrodes attached and animals were at once connected to a control phase respirator using 100% oxygen at 50 ± 5 respirations per minute and immersed in ice bath. Time required for cooling was recorded. Electrocardiographic tracings, rectal temperatures and pulse were noted continuously and records made at 5-minute intervals or less. No surgical procedures were carried out.

Results. The pulse diminished approximately 12 beats per minute for each degree C of cooling of dogs down to approximately 16°. At lower temperatures, the rate of slowing could not be correlated with rate of cooling. Variable periods of asystole occurred when the pulse was 10 per minute or less and only by electrocardiographic observations could the heart be determined to still be active.

As indicated in Table I, 8 of 11 dogs receiving cyclopropane went into ventricular fibrillation (average temp. 19.6°), 2 of 10 dogs on ether fibrillated (average temp. 18.9°), while none of the dogs receiving pentobarbital showed ventricular fibrillation. As might have been expected, animals that fibril-

TABLE I. Terminal Cardiac Event in Dogs Receiving Different Anesthetic Agents.

	Cyclopropane	Ether	Pentobarbital sodium
No. of dogs	11	10	10
Asystolic deaths	3	8	10
Mean rectal temp., °C	9.3	12.0	11.3
Mean cooling time, min.	107	100	110
Ventricular fibrillation	8*	2†	0‡
Mean rectal temp., °C	19.6	18.2	
Mean cooling time, min.	63	54	

*-† $p < .001$ Significant
*-‡ $p < .05$ ”
†-‡ $p > .05$ Not significant

lated died at higher temperatures than those which died of asystole. There were considerable differences in muscular activity of the 3 groups of animals. Animals receiving cyclopropane tended to be relatively active even though eye signs indicated that a stage of surgical anesthesia had been reached. Gross movements and even respiratory efforts appeared when the cyclopropane was gone at temperature below 28°. The animals that received ether showed some muscular action at temperatures below 28° but these movements were not marked. Animals receiving pentobarbital were quiet and showed no shivering or other muscular action after being cooled.

The 3 animals in the cyclopropane group dying of asystole expired at a mean temperature of 9.3°, the 8 asystolic ether deaths occurred at an average temperature of 12° while the asystolic pentobarbital deaths occurred at an average of 11.3°.

Discussion. It has been pointed out that an anesthetic agent or relaxing agent is necessary during the early phase of cooling to prevent shivering or other protective movements by the dog which inhibit body cooling. Haterius and Maison(2) suggested that the ideal anesthetic agent should control muscle activity and shivering until cold narcosis itself was sufficient for such control. They stated that cyclopropane was valuable an an anesthetic agent but were indefinite as to the number of animals used with cyclopropane or the average temperature to which they could be cooled successfully. One dog survived cooling to 11.7°. They also reported that dogs cooled during ether anesthesia could withstand cold better than those receiving sodium pentobarbital(3). Artificial respiration was given "as needed." In studies on effect of age on cooling, Maguire and Merendino(4) observed that adult dogs receiving 60 mg/kg of sodium pentobarbital died at average temperature of 20.4°. Eleven of these 15 died of ventricular fibrillation. Using young dogs, the average temperature of death was 12.6°, all from asystole. It appears that both youth and lack of occurrence of ventricular fibrillation influenced temperatures of death. Covino *et al.* (1) observed that 16 out of 19 dogs anesthetized with pentobarbital died of ventricular fibrillation. Those results would seem perhaps different from ours. No information, however, was given as to age of their animals. Judging from the weights (6.7 to 17.5 kg) it might be assumed that their dogs were older animals. Covino's group used push-pull artificial respiration with room air only below 26 to 24°C while our animals received hyperventilation with oxygen continuously. Our experiments, those of Maguire and Merendino(4), and of Covino *et al.*(1) all indicate that the temperature of animals dying of ventricular fibrillation is higher than that of those whose hearts have an asystolic termination of action.

Animals receiving pentobarbital undoubtedly have a considerable amount of the drug left in their bodies at the time of hypothermic deaths. Animals receiving ether probably have most of it eliminated when death occurs, but due to high solubility of ether in water and the progressively decreased circulation in hypothermic animals, some of the ether is undoubtedly left in the system. Cyclopropane is much more volatile than ether and is less soluble in water. It therefore may be assumed to be almost entirely gone from tissues by the time that cold narcosis has appeared. Anesthetization with cyclopropane followed by its practical elimination might be expected to diminish the tendency to ventricular arrhythmias, if the concept that deeper cyclopropane anesthesia increases the incidence of such irregularities(5) is accepted. Efficient concomitant elimination of carbon dioxide by assisted respiration is also said to prevent or abolish such arrhythmias(5). The use of cyclopropane, however, did not afford protection against ventricular fibrillation in the hypothermic dog, although those animals receiving cyclopropane which did not fibrillate reached the lowest temperatures of all those observed. The results obtained indicate that sodium pentobarbital may have had a protecting effect on the cardiac irritability while cyclopropane showed no such effect. The effects of ether were somewhat intermediate between those of the other two agents.

Summary. The terminal cardiac events of 31 hypothermic dogs, 6 to 12 months old, were observed. Eleven animals received cy-

clopropane and 10 each received ether and pentobarbital sodium as the pre-hypothermic anesthetic agents. All animals were hyperventilated with oxygen after intubation until the experiments were terminated. Rectal temperatures were continuously observed and electrocardiograms were taken at frequent intervals as the dogs were cooled until cardiac activity ceased. No surgical procedures were carried out. Eight of 11 animals receiving cyclopropane, 2 of 10 receiving ether, and none of 10 receiving pentobarbital died in ventricular fibrillation. The mean rectal temperature of animals on cyclopropane or ether dying of ventricular fibrillation was 19°C. The mean rectal temperatures of all the deaths after the use of cyclopropane was 16.7°, after ether, 13.4°, and after pentobarbital sodium, 11.3°.

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Effect of Malonate and Antimycin A on Renal Tubular Transport of p-Aminohippurate.*† (22024)

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The dependence of renal tubular transport systems on certain energy producing reactions might be established by the use of specific metabolic inhibitors. There exists, for example, a correlation between the capacity of certain chemical compounds to inhibit the oxidation of succinic acid *in vitro* and to decrease the renal tubular excretion of p-aminohippurate (PAH) and phenolsulfonphthalein (PSP) *in vivo*(1). In the present report additional data are presented to support the hypothesis of Shideman and Rene(1) that energy derived from succinate oxidation (and Krebs cycle oxidations) is involved in the renal tubular mechanism which effects the excretion of PAH.

Materials and methods. Female, albino rats (150-200 g) of the Holtzman strain were anesthetized by the intraperitoneal administration of 35 mg/kg of pentobarbital and the right (control) kidney was removed. Cortical slices from this kidney were prepared and approximately 100 mg of these slices were placed in 20 ml beakers containing a volume of 2.7 ml of the medium employed by Cross and Taggart(2). The concentration of PAH, however, was half that used by these authors. The

TABLE I. Effect of Malonate Administration on Ability of Renal Cortical Slices of Rat to Accumulate PAH. Each animal received 1.2 ml of M sodium malonate subcutaneously 2 hr before and 0.6 ml of the same solution one hr before removal of the left kidney (6 animals).

Rat No.	S/M concentration Before malonate (right kidney)	S/M concentration After malonate (left kidney)	% inhibition
1	8.42	2.43	80.8
2	7.81	3.59	62.0
3	6.07	2.27	75.0
4	8.39	4.07	58.5
5	10.10	8.19	21.0
6	14.90	6.27	62.1
	Mean ± S.E.		59.9 ± 8.5 P <.01

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† A preliminary report was presented at the 37th annual meeting of Fed. of Am. Soc. for Exp. Biol., Chicago, Ill., April, 1953.

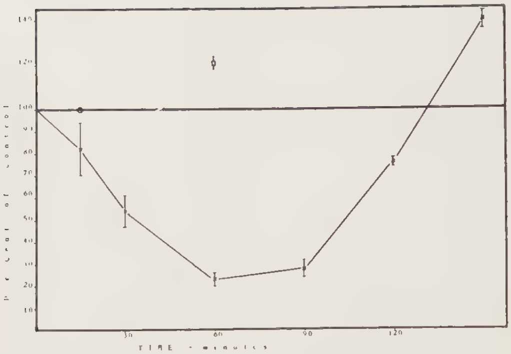

FIG. 1. Impairment of PAH accumulation by renal cortical slices from rats following administration of 1 g/kg sodium malonate. Each point with its standard error represents the data obtained from 6 animals. X—malonate, □—saline, isosmotic with and equivalent in amount to malonate administered.

beakers were incubated for one hour at 25°C in a Dubnoff Metabolic Shaking Incubator with continuous oxygenation. Following incubation, the slices were removed from the medium, weighed, and the concentration of PAH in the slice and medium determined. The capacity of the cortical slices to accumulate PAH was expressed as the ratio of the concentration of PAH in the slice to that in the medium (S/M ratio). Following removal of the right kidney, as mentioned above, sodium malonate or antimycin A‡ was administered either subcutaneously, intraperitoneally, or intravenously and at varying periods of time thereafter the left kidney was removed from each of the test animals and the capacity of cortical slices to accumulate PAH was determined. Antimycin A was dissolved in either polyethylene glycol 200 or propylene glycol for injection and in 95% ethyl alcohol for addition to the medium in the *in vitro* experiments.

Results. Following subcutaneous administration of sodium malonate in the dosage indicated in Table I there is a significant decrease (59.9%) in capacity of the kidney to concentrate PAH. Dosage schedule of sodium malonate in this representative experiment is identical with that found by Busch and Potter(3) to inhibit *in vivo* metabolism of succinate by rat kidney. In other animals which received an equivalent amount of saline, isosmotic with malonate solution employed, there was no significant alteration in the capacity of cortical slices to accumulate PAH.

The reversible nature of the inhibitory effect of malonate on transport of PAH is apparent from the data presented in Fig. 1. Sixty minutes after administration of malonate the peak effect was observed. Inhibition of this renal excretory mechanism was not demonstrable for more than approximately 2 hours after administration of malonate. Two and one-half hours following administration of the inhibitor, there was noted an appreciable increase in capacity of renal cortical slices to accumulate PAH. Intravenous saline (isosmotic with malonate) produced no inhibition 15 minutes after administration. Sixty minutes after saline, however, there was observed a slight increase in the capacity of renal cortical slices to accumulate PAH. Since the S/M ratios were based on wet weight of renal tissue, the possibility existed that this slightly enhanced capacity to accumulate PAH might be due to alteration in water content of the kidney following injection of hypertonic saline. However, no significant differences in dry weights of renal slices before and after intravenous injection of hypertonic saline were found.

The influence of varying the dosage of malonate, administered intravenously, is illustrated in Fig. 2. At doses between 0.6 and 1.0 g/kg a linear relationship was found to exist between the intravenous dose of malonate

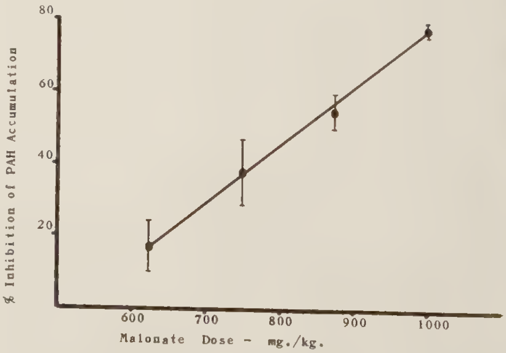

FIG. 2. Relationship between dose of sodium malonate and inhibition of PAH accumulation by renal cortical slices of the rat.

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‡ Kindly supplied by Dr. F. M. Strong, Department of Biochemistry, University of Wisconsin, Madison.

TABLE II. Inhibitory Effect of Antimycin A on Accumulation of PAH by Renal Cortical Slice of the Rabbit. Slices were prepared from cortical portion of the kidney and incubated for one hr. Antimycin was dissolved in 95% alcohol and added in a volume of 0.1 ml to each flask. Slices incubated in a medium containing the same amount of alcohol but with no antimycin served as controls.

μg antimycin A/g tissue	% inhibition
2	5.8
4	13.5
6	43.8
8	63.5
10	68.5
100	82.6
1000	91.1
1500	97.4

and percentage inhibition of uptake of PAH.

Addition of antimycin A to a medium containing slices of renal cortex interferes with their capacity to concentrate PAH (Table II). These data are from a representative experiment in which the rabbit was employed to obtain sufficient tissue to determine the effects of a fairly wide range of concentrations of the inhibitor. Similar findings obtained in the rat. The succinoxidase activity of rat kidney is inhibited by approximately 2 to 7 μg of antimycin A per gram of tissue(4). In the same concentration range, antimycin A also inhibits the accumulation of PAH by renal slices of the rabbit.

In Table III are shown the effects of parenteral administration of antimycin A on accumulation of PAH by renal slices obtained from animals treated as described below. Administration of antimycin A produced marked inhibition of PAH uptake. Reif and Potter (5) reported that comparable doses significantly suppress succinoxidase activity in kidney of the rat. Animals receiving only propylene glycol intraperitoneally or 0.5% saline and polyethylene glycol 200 intravenously served as controls. These procedures produced no significant effect on capacity of cortical slices prepared from such animals to accumulate PAH. Administration of saline prior to the polyethylene glycol 200 was effective in preventing small but significant effects produced by the latter compound when it was injected intravenously.

Discussion. In previous studies metabolic inhibitors have been used to establish the dependence of a given physiological function on specific energy yielding reactions. 2,4-Dinitrophenol (DNP), a compound which is believed to uncouple aerobic oxidative and phosphorylation reactions, has been shown by Taggart and Forster(6), and Mudge and Taggart(7) to inhibit the renal tubular excretion of PAH, diodrast, and phenol red. Metabolic blockade at the level of the Krebs cycle, with either dehydroacetic or malonic acids(1) or inhibition of oxidative phosphorylation by DNP(7), does not indiscriminately influence all renal tubular functions. These observations suggest that there is some specificity in the allocation of energy derived from oxidative mechanisms and breakdown of phosphate bond compounds for the various renal tubular functions. Although metabolic inhibitors might produce a specific biochemical lesion *in vitro,* the interpretation that their *in vivo* action is a reflection of their *in vitro* effect might be questioned. Sodium malonate and antimycin A were chosen because of the evidence for correlation of *in vivo* and *in vitro* inhibitory effects. Busch and Potter(3) have presented evidence for the *in vivo* inhibition of succinate metabolism by malonate. The same concentration of antimycin A which Reif and Potter(5) have found to inhibit succinoxidase activity in the kidney, also produces an inhibitory effect upon the renal transport of PAH. Therefore, our data, correlated with those of the above authors, are highly suggestive that succinate oxidation (and Krebs cycle oxidations) functions *in vivo* as a source of energy for this transport mechanism.

Availability of energy derived from the oxidation of malonate for the transport of PAH

TABLE III. Effect of Antimycin A Administration on Accumulation of PAH by Renal Cortical Slice of the Rat. Following removal of control (right) kidney,(a) antimycin A (1 mg/cc in propylene glycol) was administered, after 40 min. the left kidney was removed or(b) 4 cc/kg of 5% saline was administered, followed in 30 min. by intravenous injection of antimycin A (1 mg/cc in polyethylene glycol 200) 15 min. after which the remaining kidney was removed.

Route	No. of animals	Dose, mg/kg	Inhibition of PAH accumulation (% ± S.E.)
Intraper.(a)	6	3	63.4 ± 10.5
Intraven.(b)	6	1	91.4 ± 1.5

has to be considered as a possible explanation for the capacity of the renal slice to accumulate the latter material 2½ hours after the administration of malonate. Busch and Potter (3) were able to recover only part of the malonate from the urine of animals receiving this compound. It is known that malonate can be oxidatively metabolized *in vivo*(8,9) and, therefore, may contribute to the pool of metabolites from which the excretory mechanism for PAH derives its energy. Furthermore, it is possible that malonic acid is metabolized or removed from the cell more rapidly than is succinate. Succinate (and other members of the Krebs cycle) which accumulate in the presence of malonate would be expected to be present in amounts greater than are usually found and supposedly would provide an additional source of energy for the transport of PAH. Whether or not either of these proposed mechanisms is correct remains to be determined.

Summary. The renal tubular excretion of PAH in the rat is inhibited by the *in vivo* administration of sodium malonate or antimycin A. The data here presented, when correlated with the results of other investigators, strongly suggest succinate oxidation can provide an *in vivo* source of energy for that renal mechanism which is responsible for the tubular excretion of PAH.

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Effects of Digitoxin on Exchangeable and Tissue Potassium Contents.* (22025)

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The effect of digitalis on both normal and abnormal myocardium is well known. Although it is an established fact that the glycosides have a direct effect on the heart, controversy still exists as to whether they have an *extracardiac* effect as well. Because the chemical structure of digitalis is similar to that of other steroid compounds which are known to affect salt and water metabolism—for example, sex and adrenal cortical hormones, it might be suspected that the glycosides would act similarly. There are, in fact, experimental evidences to suggest that these agents, under certain circumstances, act in a fashion similar to the adrenal cortical hormones(1,2).

A previous study(3) suggested that the intravenous administration of 0.2 mg of digitoxin per kg of body weight results in reversible shifts in the distribution of body fluids. The availability of radioactive potassium (K^{42}) has made possible the direct measurement of the exchangeable potassium con-

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K^{42} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn., on allocation from the U. S. Atomic Energy Commission.

tent in intact animals. The purposes of the present study were (1) to determine the effects of large daily doses of digitoxin (0.1 mg/kg) on the exchangeable potassium content in normal rabbits, and (2) to determine the effect of a single large dose of digitoxin (0.2 mg/kg) on the tissue potassium content.

Material and methods. Sixteen normal male adult rabbits of mixed breeds were used; they were kept in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction. In order to acclimate the animals to their new environment, they were placed in the cages one week prior to the initiation of the baseline studies. Commercial crystalline digitoxin dissolved in a 40% solution of alcohol was employed. *Experiment 1.* Direct measurements of the exchangeable potassium content (Ke) were made with K^{42} at weekly intervals in 8 animals. Starting on the 8th day and continuing every day for a week, 0.1 mg of digitoxin per kg body weight was given intravenously to each animal. No digitoxin was given during the third week. The initial Ke determination was made on the first day of the experiment; the second, on the 7th day. In the analysis of the data, the mean of these two determinations was used as the baseline value for each animal. Subsequent Ke determinations were made at weekly intervals. *Experiment 2.* Each of 4 animals was given an intravenous injection of 0.2 mg of digitoxin per kg of body weight through a marginal vein immediately prior to the administration of K^{42}. Another group of 4 animals were not given digitoxin and served as controls. Twenty-four hours after intravenous injection of the radiopotassium, the animals were killed by exsanguination and analyses for radiopotassium and for total water content were performed on 18 different tissues.

Measurement of the exchangeable potassium content (Ke). The preparation of the potassium solution for injection has been previously described(4). Five ml of the potassium solution, containing 30 microcuries of K^{42} in 0.5 meq, was injected intravenously. The urine was collected for 24 hours; a spot specimen was then obtained by catheterization and its specific activity was determined. The following formula was used to calculate the value for the exchangeable potassium content of the body: $\mathrm{Ke} = \dfrac{\mathrm{Ki}^{42} - \mathrm{Ko}^{42}}{\mathrm{Ku}^{42}/\mathrm{Ku}^{39}}$, in which Ki^{42} = quantity of radiopotassium administered (arbitrary units), Ko^{42} = quantity of radiopotassium excreted in the urine in 24 hours, Ku^{42} = concentration of radioactivity in the spot specimen, Ku^{39} = concentration of total inorganic potassium in the spot specimen, and $\mathrm{Ku}^{42}/\mathrm{Ku}^{39}$ = specific activity of the spot specimen. The potassium concentration in the urine and in the serum was determined by flame spectrophotometry. *Tissue analyses.* Three portions (1 to 3 g) of each tissue were obtained. They were kept in covered Petri dishes and were weighed as rapidly as possible on a Gramatic balance to an accuracy of 0.1 mg. Two portions were digested in concentrated sulfuric acid for determination of the radiopotassium concentration. The digest was made up to a volume of 20 ml and was counted with a dipping tube and a scaling circuit. The tissue content of K^{42} was expressed in counts per minute per kg of tissue (wet weight). In the calculation of the *total tissue content* of potassium, it was assumed that at 24 hr the K^{42} was in equilibrium with the non-radioactive potassium in the body, and the specific activity of the spot specimen obtained at 24 hr (K^{42} (cpm/1)/K^{39} (meq/1)) was used to calculate the total potassium content in each tissue. This value was expressed in meq/kg. *Tissue water content* was determined on the third aliquot which was dried to a constant weight at 110°C for 18 to 24 hours. *Statistical method.* The significance of the difference between the means was determined by the "t" test(5). In the first experiment, each animal served as its own control. The differences between the baseline values and the values at the end of the second and third week were calculated for each animal; the mean difference between the baseline values and each of the subsequent determinations was then obtained. The significance of the mean difference was then tested. In the analysis of the results of experiment 2, the "t" test was again used to determine the significance of the difference between the group

TABLE I. Effects of Digitoxin in Doses of 0.1 mg per kg Daily for 7 Days on Exchangeable Potassium and Serum Potassium in 8 Normal Rabbits.

	Wt (kg)	Exchangeable potassium (meq)	Exchangeable potassium (meq/kg)	Serum potassium (meq/l)
Baseline values	2.578	207.4	78.7	5.35
Wk 1*	2.669‡	172.4‡	63.2‡	4.98‡
" 2†	2.769‡	232.9	83.7	5.29

* Mean values after 7 days of digitoxin.

† Mean values 7 days after discontinuation of digitoxin.

‡ Statistically significant difference when this figure is compared with the mean baseline value: P = less than 0.02.

means obtained in the test and control groups of animals. The calculated value for "t" was compared with the value at (n-l) degrees of freedom, as obtained from standard tables, and expressed as "P". A "P" value of 0.02 or less was considered significant.

Results. Experiment 1. (Table I). During the three-week period of observation, the mean body weight of the 8 animals in this group increased progressively from 2.578 to 2.769 kg. After 7 days of digitoxin the mean exchangeable potassium content decreased significantly from a baseline value of 207.4 meq (78.7 meq/kg) to 172.4 meq (63.2 meq/kg). The serum potassium concentration at this time was significantly lower than the baseline value, having fallen from 5.35 to 4.98 meq/l. One week after discontinuation of the drug, the exchangeable potassium content had risen to 232.9 meq.

Experiment 2. (Table II). All of the tissues in the rabbits given digitoxin contained less potassium than the tissues in the uninjected control animals; however, statistically significant differences in the mean potassium contents were observed only for the diaphragm, the abdominal muscle, and the subcutaneous connective tissue. No significant differences in the tissue water contents were found between the digitoxin-injected and the normal control animals and these values have not been included in the table.

Comment. The results of the first experiment indicate that the body store of potassium is considerably depleted within a week after the administration of digitoxin in daily doses of 0.1 mg/kg is begun. This reduction in the exchangeable potassium content was reflected in the slight, but significant, decrease in the serum potassium concentration. After discontinuation of the drug, the changes in exchangeable potassium content and in serum potassium concentration were promptly reversed.

The results of Experiment 2 suggest that the decrease in the total body content of potassium which follows the administration of a single dose of 0.2 mg of digitoxin per kg of body weight is due to a generalized loss of potassium from most of the tissues and organs of the body; the magnitude of this loss was greatest and most consistent in the abdominal and diaphragmatic muscles and in the subcutaneous connective tissue.

The results obtained in the present study are similar to those reported to occur during the administration of desoxycorticosterone acetate to normal animals(6,7). The prolonged administration of desoxycorticosterone may cause a decrease in the potassium content of muscle and necrosis of myocardial fibers. These lesions cannot be distinguished from those produced by the dietary restriction of potassium. A previous study in this laboratory has shown that the administration to nor-

TABLE II. Effects of a Single Dose of Digitoxin, 0.2 mg per kg, on Tissue Potassium Content in Normal Rabbits.

Tissues or organs	Potassium content (meq/kg) (mean ± S.E.) Uninjected control animals	Potassium content (meq/kg) (mean ± S.E.) Animals given digitoxin
Quadriceps muscles	66.77 ± 4.10	50.00 ± 6.06
Gastrocnemius muscle	66.42 ± 1.54	58.39 ± 4.75
Adrenal gland	63.33 ±24.60	37.42 ±22.80
Abdominal muscle	57.03 ± 4.21	33.34 ± 2.52*
Appendix	54.62 ± 4.10	42.28 ± 5.90
Liver	48.49 ± 3.33	38.69 ± 4.32
Spleen	45.63 ± 8.67	41.75 ± 5.77
Stomach	44.97 ± 5.18	34.79 ± 4.40
Diaphragm	44.19 ± 3.83	25.62 ± 3.76*
Heart, ventricle	41.63 ± 3.40	32.58 ± 3.34
Kidney	41.30 ± 3.30	31.91 ± 4.49
Lung	39.92 ± 2.07	29.97 ± 3.88
Heart, auricle	28.90 ± 3.41	19.59 ± 3.07
Subcut. connective tissue	28.09 ± 2.43	17.49 ± 1.86*
Skin	17.47 ± 2.99	11.07 ± 1.97

* Statistically significant difference when compared with the mean control value: "P" = 0.02 or less.

mal rabbits of large doses of cortisone acetate under experimental conditions closely resembling those employed in the present study also produces a significant decrease in the exchangeable potassium content(8). The results of the present study suggest that one of the effects of digitoxin, administered in the dosages and in the manner employed for these experiments, is to deplete the body store of potassium. This depletion is thought to be the result of an extracardiac effect of digitoxin.

Summary. 1. The daily intravenous administration of 0.1 mg of digitoxin per kg of body weight to normal rabbits resulted, within 7 days, in an increase in body weight and a reduction in the serum potassium concentration and the exchangeable potassium content. These changes in potassium metabolism were corrected within one week by discontinuing the glycoside. 2. The intravenous administration of a single large dose of digitoxin (0.2 mg per kg) to normal rabbits resulted within 24 hours in a decrease in the mean potassium content of all the tissues and organs. Statistically significant decreases in potassium content were noted in the abdominal and diaphragmatic muscles and in the subcutaneous connective tissue. The results suggest that one of the effects of the administration of digitoxin in these dosages is to deplete the body store of potassium.

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A Practical Method for Removal of Anticomplementary Properties from Human Serum. (22026)

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Methods for the removal of anticomplementary (AC) qualities from sera(1-11) are generally too complex or inconvenient for frequent use in complement-fixation (CF) tests. Since Coons and his colleagues have found that absorption of fluorochrome-labeled antibody with tissue powder reduces or removes nonspecific staining activity(12,13) the effect of tissue powder on the AC properties of human sera was investigated. Sera were freed of AC activity by the relatively simple procedure of treatment with mouse liver powder.

Materials and methods. To make tissue powder, livers from normal adult white mice (Albany strain) were cut into 10- x 10-mm pieces after the removal of fat and connective tissue, and homogenized in a Waring blendor with distilled water (30 ml/100 g). The suspension was centrifuged and the supernatant removed. The sediment was washed 3 times in distilled water, then in 0.85% NaCl solution, and mixed thoroughly with 4 volumes of acetone. The acetone was removed after centrifugation and one volume of acetone was added to the sediment. The acetone was

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[*] Based on a thesis submitted by F. Rapp in partial fulfillment of the requirements for the degree of Master of Science in Union University, Albany Medical College.

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evaporated overnight at room temperature in Petri plates. Some samples were further dried in a vacuum desiccator over $CaCl_2$. *Virus and rickettsial antigens.* Virus antigens were prepared in this laboratory. St. Louis encephalitis antigen was a suspension of infected mouse brain(14) inactivated by ultraviolet radiation. The rest were made from tissue or fluids of infected embryonated hens' eggs. Eastern and Western equine encephalomyelitis antigens were extracts of embryo and chorio-allantoic membrane suspensions in allantoic fluid also inactivated by ultraviolet radiation. Herpes simplex antigen was an extract of frozen and thawed chorioallantoic membranes. Mumps antigen was dialyzed allantoic fluid. Preparation of the soluble influenza antigens was based on Hoyle's method(15). Methods for production of psittacosis(16) and lymphocytic choriomeningitis(17) antigens have been described. All the virus antigens were stored in the dry state. Rickettsial antigens were Lederle Laboratory products. *Treatment of sera.* Serum was added to the liver powder in the proportion of 200 mg of powder/ml (50 mg/1 ml of 1:4 dilution) and shaken for one hour in a wrist-action machine.‡ The mixture was centrifuged for 30 minutes at 2000-2500 r.p.m. and the supernatant removed from the sedimented liver powder with a fine Pasteur pipette, then inactivated for 30 minutes at 60°C. *Complement-fixation tests.* Treated and untreated sera were appropriately diluted and examined in a quantitative CF test(18) with one, two, and three 50% units of complement in the absence of antigen to determine anticomplementary titer. Serial 2-fold dilutions of serum, beginning with 1:4, were tested with various antigens and three 50% units of complement. Fixation for 18-24 hours at 3°-6°C was followed by incubation for 30 minutes at 37°C with sensitized cells. Antigen controls, complement controls, with one, two, and three 50% units, and a control for sensitized cells were included; and tests with treated and untreated "positive" and "negative" control sera were included with each group of anticomplementary sera examined. Titers were expressed as the number of 50% complement units fixed by undiluted serum. Sera were considered AC if two 50% units of complement induced less than 90% hemolysis in a 1:4 serum dilution in the absence of antigen. The calculated minimum number of 50% complement units fixed by the least AC sera was 5.7 according to this criterion. Many of the sera selected for study were more strongly AC.

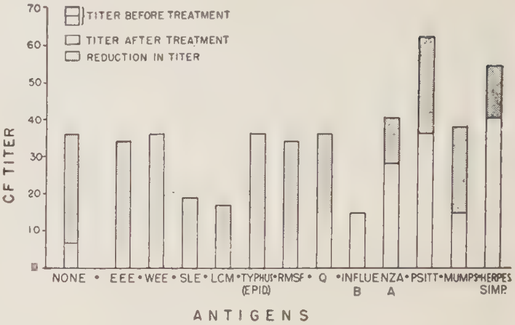

FIG. 1. Effect of liver powder treatment on strongly AC Pool I (20 sera) in CF tests with 12 different antigens. EEE, WEE, SLE = Eastern, Western, and St. Louis encephalitis; LCM = lymphocytic choriomeningitis; RMSF = Rocky Mountain spotted fever.

Results. The objects of the experiments were: To determine the extent to which liver powder removed or inhibited AC activity; to find out if specific antibody titers were altered; and to ascertain whether nonspecific CF properties were introduced by liver powder treatment. Accordingly, both AC sera and sera free of AC activity were examined in CF tests in the absence of antigen, giving the so-called "serum control titer"; in the presence of specific virus or rickettsial antigens; and in the presence of "control" antigens prepared from uninfected tissues or fluids.

Anticomplementary sera. To determine whether liver powder treatment would reduce or remove AC activity, AC sera were pooled and tested prior to and after treatment. Fig. 1 shows the results with one strongly AC pool examined with 12 virus or rickettsial antigens. The untreated serum control fixed thirty-six 50% units of complement, the treated control 6.8, a reduction of 29 units. In all cases, "control" antigens fixed <8 units of complement after treatment. The titers with 6 of the specific antigens were also reduced to <8

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‡ Burrell Technical Supply Co., Pittsburgh, Pa.

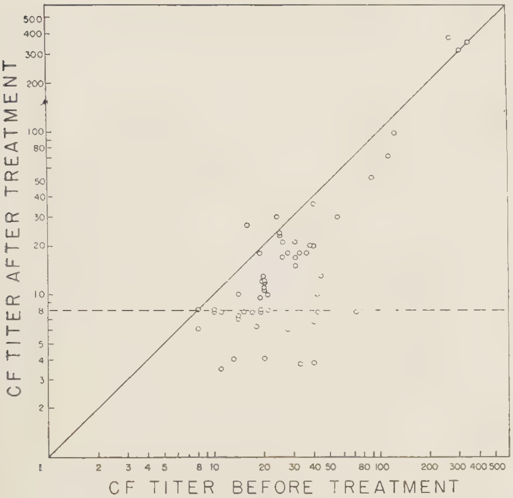

FIG. 2. Effect of treatment on 32 AC sera in 56 tests with 7 different virus or rickettsial antigens.

after treatment, a level considered nonreacting in the test employed. With 4 other antigens, however, namely herpes simplex, mumps, psittacosis, and influenza Type A, some activity remained after liver powder treatment. The reduction corresponded to that obtained in the absence of antigen. It was reasonable to assume, therefore, that the pool contained antibodies to these viruses, and that the procedure did not destroy or denature the antibody. Confirmatory results were obtained with a second moderately AC pool.

In the next experiment, untreated and treated portions of 32 individual AC sera were similarly examined. Liver powder treatment reduced the AC activity of all but one serum, and 22 of the 32 were freed of AC properties, *i.e.*, they fixed less than 5.7 fifty percent units of complement in the absence of antigen. Five of the remaining 10 sera fixed <8 fifty percent units of complement, but 4 markedly AC sera were not affected as decidedly.

To estimate further the effect of treatment on AC activity on these 32 sera, 56 tests were done with different virus or rickettsial antigens and 50 tests with their corresponding uninfected tissue or fluid control antigens. Liver powder treatment decreased the amount of complement fixed with the virus or rickettsial antigen in all but 6 instances (Fig. 2). In 80% of the sera tested, this decrease closely approximated the reduction in AC activity. Table I gives the results obtained with 5 sera from this group tested with various antigens.

Sera free of anticomplementary activity. More conclusive evidence regarding the effect of liver powder treatment on antibody was

TABLE I. Representative Changes in CF Titers of AC Sera Treated with Liver Powder.

		Titers		Titer differences
Serum No.	Test	Pre-treatment* A	Post-treatment* B	A−B†
2072	No antigen (AC activity)‡	26	12	14
	With "control" antigen§	20	14	6
	With influenza Type A antigen	36	18	18
1159	No antigen (AC activity)	18	5.3	12.7
	With "control" antigen	20	<8	>12
	With psittacosis antigen	56	30	26
2462	No antigen (AC activity)	16	5.6	10.4
	With Q fever antigen	19	<8	>11
2205	No antigen (AC activity)	19	6.4	12.6
	With "control" antigen	22	9	13
	With influenza Type A antigen	33	18	15
	Idem B	31	17	14
	" C	28	18	10
2091	No antigen (AC activity)	31	22	9
	With "control" antigen	26	21	5
	With mumps antigen	42	30	12

* Pre- or post-treatment with liver powder.
† A−B = Effect of liver powder in decreasing titer.
‡ Determination of AC activity by test in absence of antigen ("serum control").
§ Test in presence of uninfected tissue or fluid antigen corresponding to virus or rickettsial antigen.

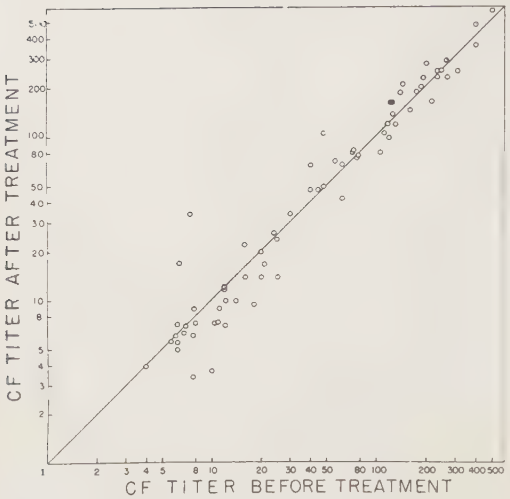

FIG. 3. Effect of treatment on 41 sera free of AC activity in 70 tests with virus or rickettsial antigens.

obtained from studies of sera that had no AC activity. Forty-one sera with titers ranging from <8 to 497 were investigated in 70 tests with virus or rickettsial antigens. Titers of these sera were virtually unaltered by liver powder treatment (Fig. 3). Only 13 (19%) pre- and post-treatment titers differed by more than 25%.

Discussion. Removal of anticomplementary activity without destruction or denaturation of specific complement-fixing antibodies has been a problem from the early days of the CF test. Sachs'(1) method of treatment with diluted HCl depends upon formation of a precipitate that carries down most or all of the AC activity. Maltaner and Maltaner(2,3) modified Sachs' technic. They adjusted the pH of sera to various levels, and concluded that the amount of AC activity removed was proportional to the euglobulin precipitated. For optimum results, it was necessary to adjust the relative concentrations of reagents for each serum. The method was therefore not suitable for large-scale application. Davis *et al.*(4) showed that the AC properties of electrophoretically separated γ-globulin were neutralized by appropriate amounts of β-globulin or albumin. They also investigated high-speed centrifugation as a method for removing AC activity, but concluded that its usefulness is limited. Taran(6) and Maaløe(7), like Davis *et al.*, attempted to neutralize rather than to remove the substances responsible for AC activity. They adopted the direct method of adding complement to replace the amount bound nonspecifically by the AC substances. This method, however, has the disadvantage of decreasing or altering antibody titers; under these conditions, mixtures of complement that are apparently identical with respect to hemolytic potency may differ considerably in their ability to combine with antigen-antibody complex(19). Like the other methods, this technic has the limitation that the appropriate dose of reagent, in this case complement, must be determined for each AC serum.

The method described here is empirical. The effect of liver powder in removing or neutralizing AC properties was studied because tissue powder treatment prevents nonspecific fluorescence(12). The treatment was also highly effective in removing or reducing the AC properties of most of the sera studied, did not reduce specific CF antibody titer in most instances, nor introduce other nonspecific properties that interfere with the CF test to an important degree. Fractionation of the powder would be a promising approach toward explanation of the mechanism of action. Other tissue powders are also being examined for their ability to remove or reduce AC activity.

Preliminary studies suggest that the effect of liver powder on the AC properties of γ-globulin is worth investigation(20). Some results indicate that the method might find application in serologic tests for syphilis with cardiolipin antigen(20). The procedure lends itself well to relatively large-scale work because it is technically simple. In particular, the effective amount of liver powder does not need to be determined separately for each serum specimen. It seems worth while, therefore, to present our data for their possible practical value before an explanation is at hand, in the hope that these observations may prompt further studies of the reaction.

Summary. A simple method for the removal of anticomplementary properties from sera by treatment with acetone-extracted, dried normal mouse liver powder is described.

This material removes nonspecific properties, does not markedly reduce or increase antibody titers with sera giving specific reactions with virus and rickettsial antigens, and does not introduce nonspecific properties in nonreacting specimens.

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Inhibition of Development of Chick Embryo Liver by Aminoguanidine.
(22027)

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Studies have been made on the nutrition, metabolism, and development of the chick embryo by injection of biologically active substances into the embryonated egg. Profound inhibition of growth with production of developmental abnormalities result from injection of certain substances(1-5), and Karnofsky(6) pointed out the value of this technic as a screening method for specific growth-inhibitors.

By extension of this technic to new classes of chemical compounds, we have observed a severe inhibition of the development of the chick embryo liver by aminoguanidine. This inhibition is rather specific and has no obvious effect on the other organs of the body under the conditions described.

Methods. Aminoguanidine sulfate (AGS)† was dissolved in double distilled water to form

TABLE I. Effect of Aminoguanidine Sulfate on Mortality, Body and Liver Weight of Chick Embryo.*

Quantity injected (mg/egg)	No. of eggs	Embryos survived	Body wt (g)†	Liver wt (mg)†
H_2O	4	4	9.89 ± .89	173 ± 26
2	14	12	8.26 ± .96	139 ± 38
5	8	7	8.58 ± .52	66 ± 39
10	14	8	8.07 ± .69	74 ± 44
15	8	8	9.26 ± .71	65 ± 62
20	14	9	7.11 ± .79	45 ± 36‡
25	8	1	6.75 ± —	— —
40-60	9	0	— —	— —

* Yolk sac injections were made on 4-day-old embryos which were harvested 10 days later.

† Mean value ± avg dev.

‡ Six of the livers at this dosage each weighed less than 25 mg.

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* The authors gratefully acknowledge the technical assistance of Mr. Gene Conway and Mr. Merle Maxwell.

† Obtained from Distillation Products Industries, Eastman Organic Chemicals Department.

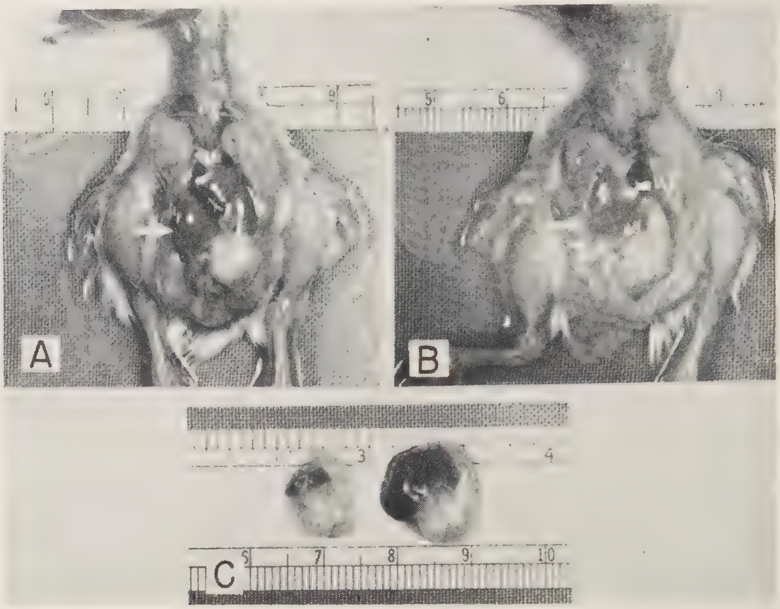

FIG. 1. Gross appearance of 14-day-old chick embryo after injection of 20 mg AGS at the 4th day of incubation. A—Control; B—Treated; C—Comparative size of livers.

a solution of desired maximal concentration with an adjusted pH of 5.6. The solution was sterilized by passage through a Selas filter of 03 porosity. Sterile double distilled water was used for further dilutions. White Leghorn eggs were incubated at 38°C and 80% humidity for 4 days. At the end of this time .4 ml of the solution to be tested was injected into the yolk according to conventional methods. After further incubation (usually until 14 days of age), the embryos were removed and examined. The specimens were preserved in 10% formalin. When histological examination was desired, the embryos of specific organs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

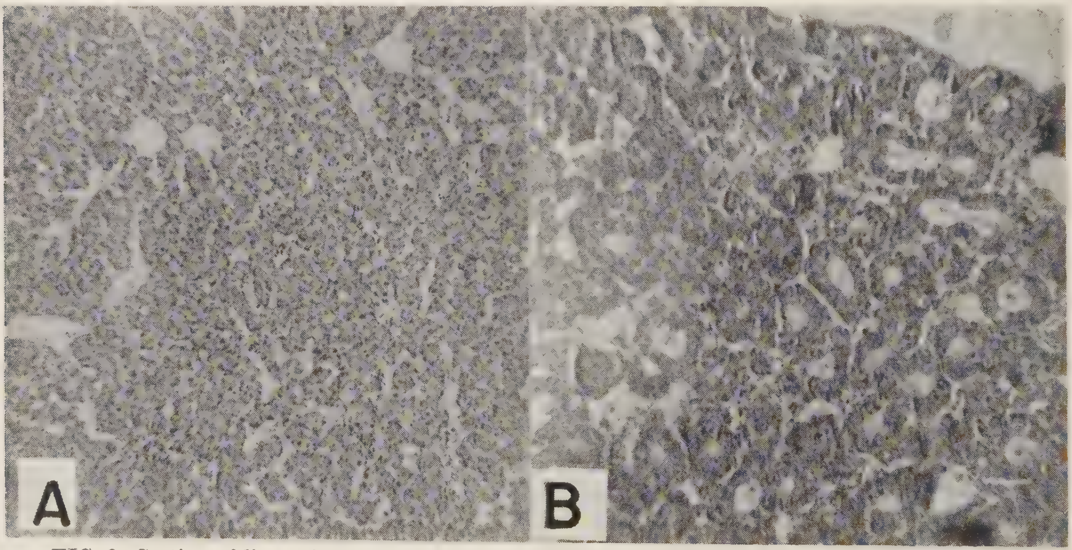

FIG. 2. Section of liver of 14-day-old chick embryo when injected at 4 days (hematoxylin eosin stain, 100 ×). A—Control; B—20 mg of AGS.

TABLE II. Effects of Aminoguanidine Sulfate on Chick Embryo when Administered at Different Embryonic Ages.*

Age at injection (days)	No. of eggs	Embryos survived	Body wt (g)†	Liver wt (mg)†
		H_2O injected		
0- 3	24	17	8.20 ± .66	155 ± 11
4-10	30	26	8.87 ± .74	175 ± 16
		2 mg aminoguanidine sulfate injected		
0	8	5	7.43 ±1.18	157 ± 11
2	8	4	— —	131 ± 25
3	6	4	8.01 ±1.89	75 ± 27
4	14	12	8.26 ± .96	139 ± 38
5	6	6	9.15 ± .96	169 ± 25
6	6	6	7.64 ± .45	161 ± 27
7	6	6	8.44 ± .73	177 ± 17
8	6	5	9.45 ± .60	195 ± 18
9	6	4	9.00 ± .70	180 ± 28
		10 mg aminoguanidine sulfate injected		
2	8	1	— —	5 —
3	6	4	7.96 ± .23	49 ± 13
4	14	8	8.07 ± .69	74 ± 44
5	6	6	8.99 ± .30	56 ± 33
6	6	5	7.70 ± .45	76 ± 19
7	6	2	7.27 ± .20	88 ± 25
8	5	2	9.00 ± .41	166 ± 25
9	6	5	8.00 ± .91	159 ± 9
		20 mg aminoguanidine sulfate injected		
2	6	—	— —	— —
3	6	2	7.32 ± .03	20 ± 3
4	14	9	7.11 ± .79	45 ± 36‡
5	12	8	7.74 ±1.02	48 ± 20
6	12	9	8.17 ± .57	95 ± 26
7	12	9	7.44 ± .41	97 ± 23
8	6	5	9.01 ± .77	141 ± 24
9	12	8	8.93 ± .79	165 ± 25
10	6	3	10.00 ± .66	182 ± 9

* Each dose was administered in 0.4 ml of solution injected into yolk. Embryos were examined at 14 days of incubation.

† Mean value ± avg dev.

‡ Six of the livers at this dosage each weighed less than 25 mg.

Results. The effect of gradated amounts of AGS on the mortality, body weight and liver mass of the chick embryo examined at 14 days of age is recorded in Table I. The number of embryos surviving 25 mg of AGS was negligible. Although the body weight at lower dosage levels was not greatly depressed, the liver mass underwent a marked reduction. At the 20 mg dosage level, ⅔ of the livers weighed less than 25 mg each and several of these consisted of only traces of tissue adherent to the duodenum. The relative mass of livers in typical treated and untreated embryos, as it appeared on gross examination, is shown in Fig. 1. The decrease in liver size and the presence of liver lesions were evident as low as 2 mg AGS dosage levels The other organs were relatively normal in gross appearance and histological sections.

The effects of 3 concentrations of AGS administered at embryonic ages varying from 0-10 days were determined (Table II). The survival rate of embryos injected prior to 4 days incubation was quite low; nevertheless, data indicate the liver to be most susceptible to inhibition when treatments are at 2-4 days. As the age of injection increased, the liver mass is decreasingly affected by a given dosage level of AGS. Finally, the liver appears perfectly normal following injection at 9-10 days of age.

In order to observe the progressive effects of AGS on the liver, eggs injected with 20 mg AGS at 4 days were removed at subsequent 48-hour intervals. Histological examination revealed that cord formation is retarded within 96 hours. In the typical liver of a treated 14-day embryo, the cord development was depressed and the parenchymal cells were replaced in large part by connective tissue and tubule-like structures (Fig. 2). Examination of serial sections revealed that these latter structures were actually oval or rounded cysts. Livers of embryos surviving beyond 14 days appeared to resume normal growth.

Discussion. Aminoguanidine has been tested on a variety of tumors and the results were considered negative(7-10). Brochman *et al.* (11) noted significant tumor inhibition by acid hydrazides such as diaminobiuret, isonicotinic hydrazide, and substituted benzoic acid hydrazides when mice bearing Sarcoma 180 were maintained on vit. B_6-deficient diet. This effect was not observed on a complete diet, but an increased restriction of tumor growth resulted when the acid hydrazides were administered in combination with desoxypyridoxine on mice maintained on a B_6-deficient diet. In the present reported studies, when pyridoxal or pyridoxine was administered in 2 mg/egg quantities concurrently with 2-4 mg/egg of AGS, no effect on the liver of the embryo was noted. Guanidine hydrochloride injected into the yolk of a 4-day embryonated egg killed all specimens at the 15 mg/egg level. Smaller quantities produced some minor liver damage but no particular abnormality as was noted in the case of AGS. An inquiry is now being made into the effect of other hydrazides upon the chick embryo. In general, these are lethal in mg doses and produce minor liver damage at lower levels. In no instance have other hydrazides produced the extreme inhibition of the liver as reported here.

Summary. Aminoguanidine sulfate when injected into the yolk of a 4-day embryonated egg produced a severe inhibition of liver development of the chick embryo when the specimens were examined 10 days later. Apparently, the parenchymal cells were the sites of inhibition as the surviving tissue, in addition to being reduced in mass, was composed largely of connective tissue and cystic structures. Other organs and tissues of the specimens appeared to be relatively unaffected. The most effective sub-lethal dose at 4 days incubation was ineffective when administered at 9-10 days of incubation. Other hydrazides possess peculiar effects which bear no resemblance to that of AGS.

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Development of Hypertension and Cardiovascular-Renal Lesions During Adrenal Regeneration in the Rat.* (22028)

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During a study of the effect of bilateral adrenalectomy on the production of hypertension and cardiovascular-renal lesions by methylandrostenediol in the rat it was observed that one control animal developed hypertension. Autopsy showed that the right adrenal of this rat had been completely removed but that a large regenerated adrenal cortex was present on the left side attached to the adrenalectomy scar. The possibility came to mind that the hypertension in this animal may have been the result of excessive hormonal secretion by the regenerating adrenal tissue. The following experiment was performed to test this hypothesis.

Methods. Twenty-four female Sprague-Dawley rats averaging 62 g body weight were unilaterally nephrectomized and given a diet of Purina Laboratory chow and 1% sodium chloride solution *ad libitum.* Twelve rats served as controls and 12 were unilaterally adrenalectomized with contra-lateral adrenal enucleation(1). This procedure consists of a small incision in capsule of the gland through which the bulk of glandular tissue is extruded by gentle application of pressure with curved forceps. The capsule and some adherent cells of the zona glomerulosa are

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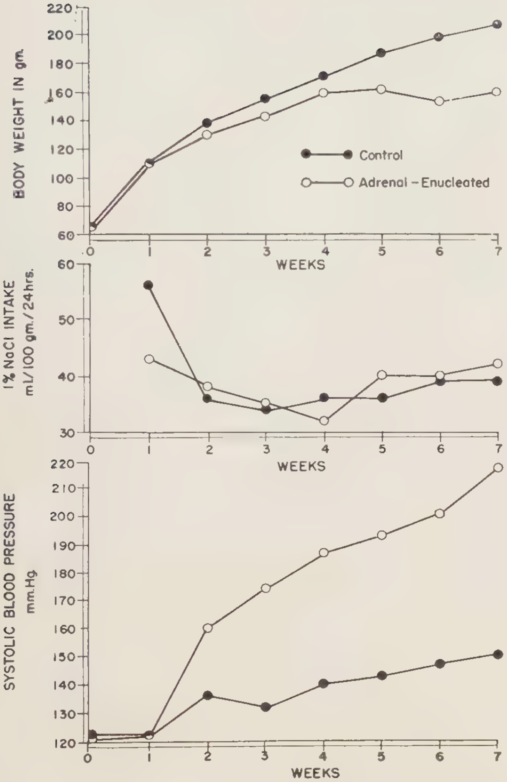

FIG. 1. Body wt, 1% sodium chloride consumption and systolic blood pressure of rats killed at 7 wk.

thus left *in situ*. Body weight, systolic blood pressure(2) and average 24-hour saline consumptions were determined weekly. Six rats in each group were killed at 7 weeks, the remaining 6 in each group at 14 weeks. At each time-interval blood was taken from the abdominal aorta and serum sodium and potassium determined on the Beckman DU flame photometer and chloride estimations made by the method of Schales and Schales(3). Organs were removed, weighed fresh and fixed in either Zenker-formal or 10% formalin. Paraffin sections were stained with hematoxylin and eosin, and frozen sections of adrenals were stained with Sudan IV.

Results. The adrenal-enucleated rats grew slightly more slowly than the controls and at no time did they consume significantly more saline solution than control rats (Fig. 1 and 2). Despite absence of an increased sodium chloride intake the adrenal-enucleated rats developed hypertension beginning from the second to fourth week (Fig. 1, 2). Systolic blood pressure of adrenal-enucleated rats killed at 7 weeks reached a terminal maximum of 217 ± 9 mm mercury as compared to terminal maximum of 151 ± 6 mm mercury in control rats. The systolic pressure of the adrenal-enucleated rats killed after 14 weeks levelled off at approximately 200 mm mercury at 9 weeks and had a terminal maximum of 197 ± 16 mm mercury as compared to 145 ± 7 mm mercury in control animals. In Tables I and II it can be seen that the serum sodium, potassium and chloride values in the adrenal-enucleated rats did not differ from the controls at either the 7- or 14-week interval.

At 7 weeks there was highly significant enlargement of the heart, kidney and brain in the adrenal-enucleated rats (Table I). The weight of the regenerated adrenal cortex of these rats did not differ from the combined weight of the adrenals in control animals. The

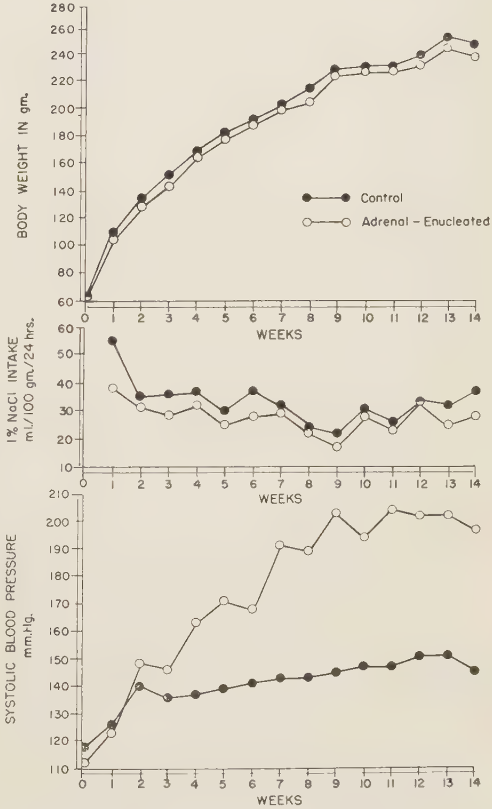

FIG. 2. Body wt, 1% sodium chloride consumption and systolic blood pressure of rats killed at 14 wk.

TABLE I. Organ Weights and Serum Electrolyte Values of Rats Killed at 7 Wk.

	No. of rats	Organ wt (mg/100 g body wt) Heart	Kidney	Brain	Adrenal(s)	Liver	Thymus	Spleen	Pituitary	Serum electrolytes (mEq/l) Na	K	Cl
Control	6	420 ± 9*	733 ± 36	823 ± 33	31.8 ± 1.0	4120 ± 220	177 ± 17	272 ± 6	4.6 ± .17	143.5 ± 0	4.42 ± .2	106.6 ± 3.4
Adrenal-enucleated	6	588 ± 31	1018 ± 108	1228 ± 34	29.0 ± .6	4987 ± 528	94 ± 22	316 ± 57	4.5 ± .4	145.2 ± 1	4.72 ± .3	110.0 ± 3.2

* Stand. error of the mean.

TABLE II. Organ Weights and Serum Electrolyte Values of Rats Killed at 14 Wk.

	No. of rats	Organ wt (mg/100 g body wt) Heart	Kidney	Brain	Adrenal(s)	Liver	Thymus	Spleen	Pituitary	Serum electrolytes (mEq/l) Na	K	Cl
Control	6	360 ± 13*	622 ± 44	716 ± 10	26.4 ± 1.2	3093 ± 109	107 ± 16	210 ± 18	4.6 ± .3	145.3 ± .9	3.9 ± .08	108.1 ± 1.8
Adrenal-enucleated	5†	442 ± 29	798 ± 47	753 ± 23	18.2 ± 1.6	3920 ± 249	92 ± 15	221 ± 28	5.6 ± .14	141.2 ± 2.0	4.2 ± .3	108.7 ± .8

* Stand. error of the mean.
† One rat died before termination of the experiment.

thymus was significantly atrophic but nevertheless present in all animals bearing regenerating adrenals. No lesions were observed in control rats but in the adrenal-enucleated animals all 6 showed renal lesions, 5 showed cardiac lesions and 5 had periarteritis nodosa in either the pancreas or mesentery or both. Cerebral changes were observed in 5 adrenal-enucleated rats and consisted of focal cerebral hemorrhages and neuronal degeneration, edema and cyst formation with bulging of the cranial vault. Microscopically the renal and cardiac lesions and periarteritis nodosa were identical to those regularly produced by overdosage of desoxycorticosterone acetate(4, 5,6), and, except for the presence of periarteritis nodosa, are remarkably similar to the lesions of malignant hypertension in the human being.

In animals sacrificed after 14 weeks, the weights of kidney and heart of the adrenal-enucleated rats were significantly greater than in the controls (Table II) but less markedly than at 7 weeks. There was no cerebral enlargement or edema in the rats bearing regenerated adrenals. The weight of the regenerated adrenal gland was now significantly less than the combined weight of the 2 adrenals of the control animals, and the weight of the thymus of adrenal-enucleated rats was the same as that of controls. Although some control rats had developed scattered glomerular lesions similar to those in the enucleated rats, the lesions were more widespread and more severe in the latter animals. In general, however, the lesions in the adrenal-enucleated animals were less severe at 14 weeks than at 7 weeks. For example, mesenteric and pancreatic periarteritis nodosa was found in only one rat and no cerebral lesions were detected.

Discussion. Considerable evidence has been presented in support of the hypothesis (4) that derailment of the General-Adaptation-Syndrome involving hypersecretion of the adrenal cortex may lead to malignant hypertension and cardiovascular-renal lesions in the human being. This theory has been largely based on experiments in rats in which adrenocortical hyperfunction has been produced by exposure of the organism to non-specific stress(7), the administration of either anterior hypophyseal trophic substances(8), or corticoid hormones(9). Because the factor of non-specific stress has not seemed to play a prominent part in human hypertension and cardiovascular-renal disease many investigators have remained unconvinced of the importance of adrenocortical hyperfunction in their pathogenesis(10,11). Without assuming that there must be such a pathogenetic relationship, as outlined above, it is considered of some importance that the present experiment has demonstrated that hypertension and cardiovascular-renal lesions can be produced apparently as a result of hyperfunction of the adrenal cortex under conditions in which exogenous non-specific stress seems to play no part. The stimulus to both regeneration and to hyperfunction appears to lie entirely within the organism, and on the basis of extensive studies by Ingle(12) is assumed to be the lowered corticoid blood levels produced by the hemi-adrenalectomy and contra-lateral adrenal enucleation which in turn leads to increased secretion of ACTH by the anterior hypophysis.

Some thought concerning the type of secretion of the adrenal cortex under these conditions may be pertinent. Ingle has shown(12) that continuous administration of ACTH to adrenal-enucleated rats can cause such profound weight loss that the animal is brought to the point of death, presumably as a result of the greatly increased secretion of gluco-corticoids. Furthermore cortisone has been shown to produce hypertension and cardiovascular-renal changes similar to those observed in the present experiment(13,14). However, this steroid produces increased consumption of sodium chloride drinking solution, profound thymic and splenic atrophy and does not cause the production of periarteritis nodosa but rather promotes the healing or prevents the development of such a lesion(13, 14). In this experiment no polydipsia occurred, thymic atrophy was never profound and present only at the 7-week interval, splenic atrophy did not occur and periarteritis was produced. Of further interest is the total absence of any significant changes in the serum electrolyte pattern.

All these observations suggest that the regenerating adrenal cortices were not secreting increased quantities of gluco-corticoids alone but rather that there was increased secretion of some mineralo-corticoid either alone or together with gluco-corticoids. Such a possibility is entirely compatible with the observations of Singer and Stack-Dunne(15), who demonstrated that the rat adrenal can respond to ACTH by liberation of both corticosterone and aldosterone into the adrenal vein. Furthermore it must be borne in mind that enucleation removes the zones which are presumed to secrete gluco-corticoids but leaves the zona glomerulosa which is considered to be the source of mineralo-corticoids, a situation which in itself might lead to imbalance of corticoid secretion. Regardless of how adrenocortical function may be altered in the present experiment the method makes possible future studies of the steroid secretory pattern of the adrenal cortex under conditions of increased function in the absence of superimposed exogenous stress and in which hypertension and vascular disease are a natural consequence.

Summary and conclusions. This report presents a simple technic for the production of a new type of experimental hormonal hypertension with cardiovascular-renal lesions in rats sensitized by unilateral nephrectomy and salt administration. It demonstrates that such hypertension and cardiovascular-renal lesions can be produced without polydipsia and excessive sodium consumption and without alteration in the serum levels of sodium, potassium or chloride. The results suggest that regenerating adrenal cortical tissue secretes increased quantities of cortical hormones and lend strong support to the hypothesis that increased adrenal cortical function is of fundamental importance in the pathogenesis of hypertension and cardiovascular-renal disease.

The author wishes to thank Dr. William Harms and Mr. Nelson Brown for the determinations of serum sodium, potassium and chloride, and to gratefully acknowledge the technical assistance of Miss Anne Dye.

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Gonadotrophic Hormone Excretion of the Pregnant Monkey (*Macaca mulatta*).* (22029)

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The object of this study was to re-examine the reports that gonadotrophic hormone is excreted by the pregnant monkey for an exceedingly limited period of time. To determine simultaneously the type of gonadotrophin excreted by the monkey, the hypophysectomized rat was used to assay the urine. The first precise evidence for excretion of gonadotrophin by the pregnant monkey was furnished by Hamlett(1), who reported a positive Friedman reaction from urine collected between the 19th and 26th days. No urine collected after the 26th day of pregnancy gave a positive test. Delfs(2) found that gonadotrophic hormone could be detected (by the rat uterine-weight test) in the blood serum of pregnant monkeys as early as the 14th day of pregnancy; the amount reached a peak by the 20th to 25th day and the hormone disappeared by the 30th day. In both of these studies the time of gestation was accurately known from cycle and breeding dates. It appears that monkeys resemble man in that gonadotrophin is present in blood and urine during pregnancy, the hormone appearing in blood and urine soon after the establishment of the placenta; however, in the macaque, the hormone appears to persist for only a short period, whereas in apes (3-5) and man it is present to the end of pregnancy.

Methods. Female rhesus monkeys, *Macaca mulatta,* of the Yale obstetrics colony were placed with males at noon of the 11th day of the menstrual cycle. Each day thereafter a vaginal lavage was examined for the presence of sperm. If sperm were recorded on the 12th day and this was followed by pregnancy, day 12 was considered to be the day of conception. Urine was collected at representative periods from females in which the span of gestation was thus accurately known. Animals were placed in the metabolism cage at noon, for a 24-hour period, and the date of the 1st day identified that urine specimen. The urine drained through a paper filter and was collected under toluol. It was frozen immediately on termination of the collection period. To concentrate for injection, the urine was filtered, acidified to pH 5 and precipitated in 3 times its volume of ethanol. The precipitate was

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TABLE I. Occurrence of Chorionic Gonadotrophin in the Urine of Seven Pregnant Macaques. As demonstrated by ovarian histology in hypophysectomized female rats.

Days	Mm 485 1954	Mm 485 1955	Mm 639	Mm 736	Mm 416	Mm 666	Mm 637	Mm 615
22	+		+					
23								
24	(toxic)		+*					
25				—				
26			+					
27	(toxic)							
28			—		+			
29								
30			+	—				
31	—	+*			—			
32		+*	—	—				
33	—	+*			—			
34		+*	—					
35					—			
36	—		—					
37								
38			—					
39					—			
40			—					
42	—		—					
44			—					
46						—		
48			—					
49	—							
51						—		
56	—				—			
62							—	
63					—			
71					—			
73							—	
76	—							
88						—		
120								—
177								—
173-179							—	

* Also gave a positive A-Z test in one normal rat (large follicles and corpora lutea) at a total dose equivalent to ¼ day's output.

washed with 95% ethanol, dissolved in water and dialyzed. Any insoluble material present after dialysis for 24 hours was removed by centrifugation and the clear supernatant was lyophilized. The powder thus obtained from the 24-hour urine sample was extracted by 16 cc water, and 1 cc aliquots were injected, subcutaneously, daily for 4 days, into each of 3 hypophysectomized female rats (26-28 days of age at operation, 8 days postoperative). Each rat, therefore, received the equivalent of ¼ day's urine output. Autopsy was performed 96 hours after onset of the injections. Before use in assay the rats were screened for completeness of hypophysectomy, and at autopsy the success of the operation was further checked so that no data from incompletely hypophysectomized rats were included. The ovaries and uteri were examined carefully under a binocular and weighed. The ovaries were subjected to microscopic analysis for study of the type of gonadotrophin present. Thyroids and adrenals were also examined microscopically for evidence of the presence of thyroid and adrenal stimulating substances in the urine. The width of the uncalcified portion of the proximal epiphyseal cartilage of the tibia was measured for evidence of growth-promoting substance.

Results. (Table I). The chief gonadotrophic effect of the pregnant monkey urine, as judged by the response of ovaries of hypophysectomized rats, was stimulation of the interstitial tissue. Urine collected between days 22 to 34 caused repair or even hypertrophy of interstitial cells when injected in the equivalent of one-fourth of a day's output. From the 35th day to the 179th day, the latest day tested, the concentrates of urine had no gonadotrophic action at the dose tested.

Follicle stimulation was not detected unequivocally in the urinary equivalents injected. An increase in the number of small follicles and some slight increase in follicle size above control level were observed on injection of urines collected between days 22 and 34. The degree of stimulation was characteristic of that seen on injection of minimal, or immediately subminimal doses of pituitary follicle stimulating hormone. There was, however, no evidence of secretion of estrogenic substances judged either from uterine weight or from vaginal reaction. Delfs(2) likewise found that the interstitial tissue of the hypophysectomized rat's ovary was the only tissue responding to the serum from monkeys in early pregnancy. It will be remembered that this selective type of ovarian reaction in the hypophysectomized rat is also typical of human chorionic gonadotrophin.

The response of the ovary of the intact rat to monkey urine gonadotrophin was compared with the well known response to human chorionic gonadotrophin by injecting normal immature rats with the urinary concentrates. One-fourth daily output of urine collected between days 24 and 34 caused the development of a few large follicles and a limited number of corpora lutea, with ovulation. The ovarian reaction in normal immature rats was, therefore, comparable to that obtained after injection of low doses of human pregnancy urine.

It should be noted that ¼ daily urinary output represented approximately 100 to 125 cc of urine. In the usual Aschheim-Zondek test of human pregnancy urine a total of only 4 cc is injected. In assay of human pregnancy urine in hypophysectomized female rats, Lyon *et al.*(6) reported follicular stimulation from ⅕ daily output of urine, or the equivalent of 200 to 300 cc per rat.

A low grade stimulation of the thyroid of hypophysectomized rats, such as is noted on injection of minimal doses of pituitary thyrotrophic hormone, was occasionally observed on injection of urines collected between the 22nd and the 177th day of pregnancy. (The thyroid epithelium was slightly taller, the nuclei were more vesicular, the cytoplasm slightly more abundant, and the colloid somewhat vacuolated and lighter-staining.) These results were therefore suggestive that small amounts of thyrotrophic hormone were present in the pregnancy urine. There was no consistent evidence for adrenocorticotrophic substance, judged either from adrenal weights or histology. Likewise, the uncalcified portion of the proximal epiphyseal cartilage of the tibia of the hypophysectomized rats was in no instance wider than that of uninjected controls, so there was no evidence of excretion of growth-promoting substance.

Summary. The urinary excretion of gonadotrophin by pregnant *Macaca mulatta* was assayed at intervals from day 22 to 179 of gestation. Hypophysectomized immature female rats were injected with concentrates equivalent to ¼ day's output of urine. Only urine excreted between days 22 and 34 of pregnancy had a gonadotrophic action, and the action was restricted to repair of the interstitial tissue. The gonadotrophic stimulus observed in normal immature rats was comparable to that from low doses of human chorionic gonadotrophin during this extremely limited period of pregnancy.

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Comparative Effects of Dietary Sucrose and Cornstarch on Tryptophan Requirement of the Rat.* (22030)

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Available data indicate that the growth increment of rats fed low protein rations is dependent to a considerable degree on the source of dietary carbohydrate. Krehl *et al.*(1), Henderson *et al.*(2), Harper and Katayama (3) and others(4-6) observed that dextrin or cornstarch support better growth of rats fed low casein rations than does sucrose. Similar results have been reported by Womack *et al.* (7) for rats fed a low level of an amino acid mixture. However, the differences in growth on these diets were eliminated when the casein content of rations or the level of the amino acid mixture was increased. In the present communication data are presented indicating that the source of dietary carbohydrate may also affect growth increment of rats fed a diet deficient in a single amino acid.

Procedure. Two basal rations were employed, diets A and B. Diet A was a tryptophan-free ration of the following composition in percent: sucrose, 60.8; acid hydrolyzed casein,[†] 24; cottonseed oil (Wesson), 10; salt mixture,[‡] 5; and l-cystine, 0.2. To each kg were added the following crystalline vitamins: thiamine hydrochloride, 40 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 100 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 g; vit. A, 5000 U.S.P. units; vit. D_2, 500 U.S.P. units; and alpha tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Diet B was similar in composition but contained cornstarch in place of sucrose as the dietary carbohydrate. In addition to the basal rations the following diets were also employed, consisting of basal rations A and B plus each of the following supplements: (a) 600 mg dl-tryptophan/kg diet (b) 1.8 g dl-tryptophan/kg diet and (c) 5.4 g dl-tryptophan/kg diet. These supplements were incorporated in the diet in place of an equal amount of hydrolysate and corresponded to 0.25%, 0.75% and 2.25% respectively of the hydrolysate content of the basal ration. In addition to above diets, experiments were also conducted with diets which were identical to basal rations A and B except that the acid hydrolysate was replaced by intact casein.[§] Two hundred albino female rats of the University of S. California strain, were selected at 21 to 24 days of age and average body weight of 42 g (range 36 to 50 g). Animals were placed in metal cages with raised screen bottoms and fed above diets *ad lib.* (20 rats/group). Diets were prepared weekly and stored under refrigeration. Rats were fed daily, and all food not consumed 24 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. Feeding was continued for 18 weeks or until death, whichever occurred sooner. Of the surviving animals, 12 in each group were autopsied and gonadal and uterine weights determined. Ovaries were fixed in Bouin's solution, and sections were prepared and stained with hematoxylin and eosin.

Results are summarized in Table I. Findings indicate that the weight increment of immature rats fed a tryptophan-free diet supplemented with 600 mg dl-tryptophan per kg diet was more than twice as great on

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[†] The authors are indebted to Dr. Harry Wax of the Stuart Co., Burbank, Calif., for the acid hydrolyzed casein used in this study.

[‡] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

[§] Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

TABLE I. Effects of Dietary Sucrose and Cornstarch on Body, Ovarian and Uterine Weights of Rats Fed a Tryptophan Deficient Ration Supplemented with Graded Levels of dl-tryptophan (20 Animals per Group).

Dietary group	Tryptophan supplement, g/kg of diet	Increment in body wt after 18 wk,[*] g	Ovarian wt, mg	Uterine wt, mg
S[†]	.6	57 ± 5.1	18	66
C	.6	128 ± 5.2	45	348
S	1.8	152 ± 4.1	42	300
C	1.8	148 ± 6.0	51	380
S	5.4	146 ± 5.3	49	263
C	5.4	151 ± 3.7	47	298
S	Intact casein	199 ± 4.9	58	398
C	" "	200 ± 6.0	64	483

[*] Including stand. error of the mean.
[†] S = Sucrose; C = Cornstarch.

the cornstarch-containing ration than on diet containing sucrose as the source of carbohydrate. Average weight increment of rats at this level of tryptophan supplementation after 18 weeks of feeding was 128 ± 5.2[‖] g in the corn starch series and 57 ± 5.1 g for animals fed the sucrose-containing diet. At higher levels of tryptophan supplementation (1.8 g and 5.4 g dl-tryptophan/kg diet), no significant difference in weight increment was observed between rats on cornstarch or sucrose-containing rations. Similarly, no significant difference in weight increment occurred between these two dietary groups when intact casein was fed in place of the hydrolysate. Animals fed unsupplemented tryptophan-free rations A and B lost weight from the start of feeding and died within 6 weeks of start of experiment. A marked difference was noted, however, between rats in the two groups. On the sucrose-containing diet animals developed an unthrifty appearance with ruffled fur and varying degrees of alopecia. On the cornstarch-containing ration, although weight loss of rats was the same as in the sucrose group, the fur remained smooth and sleek. After 28 days of feeding only 1 out of 20 rats fed the basal ration A was still alive in contrast to 16 out of 20 rats on ration B. The average survival time for rats on tryptophan-free diets was 24 ± 0.6 days (range 20 to 29 days) on ration A and 33 ± 1.3 days (range 23 to 41 days) on ration B.

At autopsy significant differences in ovarian and uterine weights were noted between cornstarch and sucrose series in rats fed a tryptophan-free ration supplemented with 600 ml dl-tryptophan/kg diet. On the sucrose-containing diet the ovaries and uteri appeared immature both in weight and microscopic appearance. These findings might have been anticipated since the corresponding organs of rats of similar weight which had been fed a natural food stock ration were also immature. In contrast rats in the cornstarch series at the same level of tryptophan supplementation had well developed ovaries and uteri[¶] in addition to increased body weight. At higher levels of tryptophan supplementation (1.8 g and 5.4 g dl-tryptophan/kg diet) and on intact casein rations, the ovaries and uteri were well developed in both the sucrose and cornstarch-containing series.

The weight increment of rats fed dl-tryptophan at levels of 1.8 g and 5.4 g per kg of diet was significantly greater both in the cornstarch and sucrose series than that of animals receiving the lower level of supplement (600 mg dl-tryptophan per kg of diet) but was still approximately one-third lower than that of rats fed the intact casein. Since the 1.8 g level of supplementation was as effective as the higher level of supplement in promoting the weight increment of rats fed the hydrolysate-containing rations, and since the weight increment of rats fed these supplements both in the cornstarch and sucrose series was significantly less than that of animals fed the intact casein, it would appear that the acid hydrolysate employed in the present experiment may have been deficient not only in tryptophan but in some other factor or factors present in the intact casein.

[‖] Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[¶] Subsequent data indicate that ovaries of rats in this group were normal functionally as well as in histologic appearance. After the 18th week of feeding, 6 animals in this series were bred to males of proven fertility. All 6 gave birth to living young which appeared normal in all respects except for size (young averaged 3.8 g at time of birth).

Discussion. Findings suggest that the source of dietary carbohydrate may profoundly affect the biological value of a protein. Rats fed a tryptophan deficient ration supplemented with a suboptimal level of dl-tryptophan (600 mg/kg of diet) grew poorly when sucrose was the dietary carbohydrate but showed a significant increment in body weight over that observed on the sucrose-containing diet when cornstarch replaced sucrose as the source of dietary carbohydrate. At higher levels of tryptophan supplementation (1.8 g or 5.4 g dl-tryptophan/kg of diet) no significant difference in weight increment occurred between rats fed cornstarch or sucrose-containing rations. Similar findings were obtained in respect to ovarian and uterine weights. Ovaries and uteri appeared infantile in rats fed a tryptophan-deficient ration supplemented with suboptimal levels of dl-tryptophan when sucrose was the dietary carbohydrate but appeared normal in size and appearance either when cornstarch replaced sucrose as the source of dietary carbohydrate or when the tryptophan content of the sucrose-containing ration was increased.

No data are available to account for the beneficial effects of cornstarch over sucrose for rats fed suboptimal amounts of tryptophan under conditions of the present experiment.** It has been suggested that essential amino acids may be synthesized by the intestinal flora of rats fed cornstarch(4) and it is possible that sufficient amounts of tryptophan may be available from this source to account for the observed results. The converse explanation—that the intestinal flora of rats fed sucrose use greater quantities of tryptophan than the intestinal flora of animals fed cornstarch—would appear, however, to be equally tenable(3). Another hypothesis is that the rate of digestion may be slower when rations containing cornstarch in place of sucrose are fed and that this may permit a more efficient utilization of tryptophan(5). Regardless of the final explanation of the observations reported above, however, findings clearly indicate that the source of dietary carbohydrate may, at least under certain experimental conditions, significantly modify the tryptophan requirement of the rat.

Summary. Immature female rats were fed tryptophan-free rations supplemented with graded levels of dl-tryptophan and containing either sucrose or cornstarch as the source of dietary carbohydrate. Rats fed dl-tryptophan at a level of 600 mg/kg of diet grew poorly when sucrose was the dietary carbohydrate but showed a significant increment in body weight over that observed on the sucrose-containing diet when cornstarch replaced sucrose as the source of dietary carbohydrate. At higher levels of tryptophan supplementation (1.8 g or 5.4 g dl-tryptophan/kg of diet) no significant difference in weight increment occurred between rats fed cornstarch or sucrose-containing rations.

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** In view of the fact that some starches contain small amounts of protein, the possibility was considered that sufficient amounts of tryptophan were derived from this source to account for the observed results. Nessler nitrogen determinations of the cornstarch employed in this experiment, however, revealed no detectable N.

Interrelationships of Methionine and Vitamin B_6 in Human Subjects.* (22031)

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(Introduced by C. A. Elvehjem.)
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That vit. B_6 is involved with the metabolism of methionine has been shown by various investigators. Miller and Baumann(1) and Cerecedo and Foy(2) found that high protein diets increased the vit. B_6 requirement. Later work indicated that the adverse effects of high protein diets are due in part to the methionine content of the protein. Methionine in excess of the normal requirement resulted in depressed growth(3-5), an effect particularly evident when the diet was low in vit. B_6(6,7). Linkswiler *et al.*(8) added only 0.4% methionine to an assay diet containing 18% casein and observed that the symptoms of deficiency of the vitamin were aggravated sufficiently that the rat assay could be conducted without a preliminary depletion period. It was observed by DeBey *et al.*(9) that while vit. B_6 counteracted the effects of moderate amounts of methionine, with 2.5% methionine, high levels of the vitamin failed to restore growth. The role of B_6 and methionine in the formation of N^1-methylnicotinamide (NMeN) from nicotinamide has been suggested by other workers. Handler and Dann(10) suggested that nicotinamide is methylated *in vivo* by methionine and excreted in the urine as NMeN. In studies on the urinary excretion of NMeN, Rosen *et al.*(11) noted that B_6-deficient rats did not excrete NMeN. When pyridoxine was administered to these animals, there was a very gradual reappearance of NMeN. Perlzweig *et al.*(12) demonstrated that NMeN is formed *in vitro* by rat liver slices from nicotinamide but not from nicotinic acid. Ellinger (13) confirmed these results and showed that the particular enzyme involved was specific for nicotinamide but not for nicotinic acid. These workers(12,13) observed that the extent of transformation was increased by the addition of methionine to the system. No report on the study of the effect of both pyridoxine and methionine in NMeN formation in man has been found in the literature.

The present investigation was designed to study the effects of pyridoxine and methionine on NMeN formation in man and the effect of a high intake of methionine on the urinary excretion of B_6 and its metabolites by subjects on a diet low in vit. B_6.

Methods. Four normal adult women participated in each experiment, 3 of them taking part in both experiments. This study consisted of 2 experiments of 21 days each, with 3 months intervening between them. Each experiment was divided as follows: a 9-day period during which the subjects received only the basal diet followed by two 6-day periods in one of which the basal diet was supplemented by 8 g of dl-methionine and in the other by 8 g dl-methionine and 10 mg of pyridoxine hydrochloride. The methionine was

TABLE IA. Basal Diet Low in Vit B_6.

Food	Wt, g	Vit B_6 (analytical value), mg	Methionine (calculated value), mg
Orange juice, frozen	100	.021	3.0
Peaches, canned	100	.018	2.0
Apple sauce	100	.001	3.0
Lettuce	10	.003	
Carrots, raw	30	.008	
Celery	5	.017	
Onion	5	.004	
Cream of wheat, dry	25	.024	58.0
Rice, uncooked	50	.045	53.8
Cottage cheese, dry	30		142.5
Gelatin	5		36.0
Biscuits*		.026	
Minerals*			
Vitamin supplements*			
Totals		.167	528.7

* Composition given in Table IB.

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TABLE IB. Basal Diet—Continued.

	g
Biscuits:	
Cake flour	100
Baking powder	8
Crisco	34
Vitamin-free casein	40
Water	70
Salt mixture:	
$CaCO_3$	1.2
$KHCO_3$	1.88
$Mg\,SO_4\,7H_2O$	.52
$Fe_2\,(C_6H_5O_7)_3\,3H_2O$	.14
$Mn\,SO_4 \cdot 4H_2O$	Trace
$Cu\,SO_4 \cdot 5H_2O$	”
KI	”
Vitamins:	
Thiamine hydrochloride	1.2
Riboflavin	1.5
Niacin or nicotinamide	10.0

divided into 3 portions, one portion being taken with each meal. Pyridoxine was taken with breakfast during the last period. A supplement of 10 mg niacin was given during the first experiment and 10 mg of nicotinamide, during the second. The *basal diet* for the entire experiment is given in Table I. This diet was found by analysis to furnish 0.167 mg of vit. B_6 per day and 58 g of protein. Forty grams of the protein came from "vitamin-free" casein which was incorporated in biscuits. By computation, the diet was found to contain 428 mg of methionine and 2.69 mg of niacin. Cookies consisting of butter, sugar and cornstarch furnished additional calories to meet the energy requirement of each individual. The intake for each subject remained constant throughout the experiment. After a 3-day preliminary period in each experiment, 24-hour urine collections were made and preserved with toluene and 5 ml of glacial acetic acid. These were pooled into 3-day periods and samples were stored in a refrigerator for analyses. The B_6 content of the food and the urine was determined using the method of Atkin *et al.*(14). The 4-pyridoxic acid in the urine was determined by the method of Huff and Perlzweig as modified by Sarett(15); NMeN was determined according to the acetone-fluorometric method of Huff and Perlzweig(16).

Results. Effect of pyridoxine and methionine on N^1-methylnicotinamide excretion. The data on the urinary excretion of NMeN are presented in Table II. The fourth subject differed in the 2 studies but the values are shown consecutively for comparison. From these data it may be concluded that the methionine supplement and the later addition of pyridoxine hydrochloride to the diet low in B_6 had no effect on the urinary excretion of NMeN when the diet was supplemented with nicotinic acid. This was observed in all 4 subjects. During the low B_6 period, when nicotinamide was the supplement, the excretion of NMeN was approximately the same as when nicotinic acid was taken. The addition of methionine alone had little effect on the excretion. However, when pyridoxine was added in the presence of methionine the NMeN excretion rose markedly for each subject. For 3 subjects the excretion during this period was equal to more than 100% of the nicotinamide supplement and for the fourth subject, it was equal to approximately 65% of the supplement.

If nicotinic acid were readily converted to nicotinamide in the body, one would expect the same effect from both supplements. The increased excretion of NMeN observed only when the supplement was in the form of nicotinamide but not when it was nicotinic acid demonstrates the difficulty in the formation of NMeN from nicotinic acid in man. These results agree with the findings of Perlzweig and his coworkers(12) and Ellinger(13), using rat liver preparations.

The role of B_6 in NMeN formation is shown

TABLE II. Average Daily Urinary Excretion of N^1-methylnicotinamide.

Subject	Basal diet, mg	Basal + 8 g dl-methionine, mg	Basal + 8 g dl-methionine + 10 mg pyridoxine HCl, mg
	Period I—Nicotinic acid supplement		
RZ	5.20	4.24	4.02
LM	4.76	5.10	5.10
KC	3.18	4.42	4.32
RB	3.56	5.57	4.74
	Period II—Nicotinamide supplement		
RZ	8.95	7.20	11.95
LM	3.75	4.50	12.75
KC	5.85	6.15	14.20
AW	5.40	5.40	6.85

TABLE III. Average Daily Urinary Excretion of Vitamin B_6 and 4-Pyridoxic Acid by Human Subjects on a Diet Low in B_6 and Supplemented with dl-Methionine and with dl-Methionine and Pyridoxine HCl.

	Subject RZ		Subject LM		Subject KC		Subject RB	
	Vit B_6	4-P.A.	Vit B_6	4-P.A.	Vit B_6	4-P.A.	Vit B_6	4-P.A.
Period 1	mg							
Basal diet*	.06	2.48	.04	1.51	.02	2.74	.04	2.72
Basal diet + 8 g dl-methionine	.04	2.69	.03	2.35	.06	2.40	.04	3.46
Basal diet + 8 g dl-methionine + 10 mg pyridoxine hydrochloride	.47	8.10	.39	6.82	.59	5.68	.60	8.17
Period 2							Subject AW	
Basal diet	.06	2.38	.05	1.98	.06	2.57	.06	2.39
Basal diet + 8 g dl-methionine	.06	2.15	.06	2.63	.06	2.60	.07	3.11
Basal diet + 8 g dl-methionine + 10 mg pyridoxine hydrochloride	.53	8.37	.47	6.99	.63	6.62	.63	6.51

* Basal diet furnished 0.16 mg B_6.

by the increased excretion by human subjects of NMeN in the presence of both pyridoxine and methionine but not of methionine alone. A more complete picture of the role of B_6 in the methylation of nicotinamide might have been obtained had there been included an additional period in which pyridoxine without methionine was the supplement.

Urinary excretion of B_6 and metabolites. Data on the urinary excretion of B_6 and 4-pyridoxic acid are presented in Table III. The 4-pyridoxic acid values have been converted to pyridoxine by using the factor 0.92 based on the molecular weights of the 2 compounds. From these data, it appears that a high intake of methionine had no effect on the urinary excretion of vit. B_6 and 4-pyridoxic acid. Similar results were obtained in both experiments. De Bey(9) reported that dietary methionine had no effect on the concentration of vit. B_6 in the blood or liver of rats.

It may be noted from Table III that the 4-pyridoxic acid excretion exceeded the intake of vit. B_6 during the first 2 periods of the study. The amount of B_6 in the diet was 0.167 mg per day, whereas the excretion of 4-pyridoxic acid and B_6 ranged from 1.55 to 3.55 mg per day. Linkswiler *et al.*(17) reported a similar elimination of B_6 and metabolites in excess of intakes of 0.78 mg and 2.78 mg per day.

The fact that the diet used in the current study was almost a synthetic one and excluded foods reported by Sarett(15) to contribute interfering fluorescent substances in the urine, makes it likely that the fluorescent substance measured was actually 4-pyridoxic acid. The 10-mg supplement given the subjects during the last period of the study accounts for the high excretion of B_6 and 4-pyridoxic acid during this period.

Summary. 1. A study of the interrelationship of methionine and pyridoxine was made in adult human subjects maintained on a semi-synthetic diet, low in vit. B_6. 2. No effect of a high dosage of methionine on the urinary excretion of B_6 and its metabolites was observed in the study. 3. The role of vit. B_6 in the methylation of nicotinamide is shown by the increased urinary excretion of N^1-methylnicotinamide by human subjects, when given methionine plus pyridoxine but not when given only methionine.

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Effect of Meningococcal Endotoxin on the Immune Response.* (22032)

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Administration of sublethal quantities of various preparations of Gram negative bacterial endotoxin produce fever, leucocytosis preceded by leucopenia, depletion of liver glycogen, alterations in blood sugar levels, vasomotor disturbances and hemorrhagic necrosis of rapidly growing tumor tissues(1,2). In addition to these systemic reactions endotoxins produce profound effects on the host's natural defense mechanisms(3-5). An intravenous injection of suitable endotoxin establishes a state of "preparation" for the generalized Shwartzman phenomenon(6), so that a subsequent intravenous injection of endotoxin results in widespread hemorrhagic necrosis in the visceral organs of rabbits. The rabbit in this "prepared state" exhibits impaired activity of the reticuloendothelial system, as measured by rate of clearance of colloidal gold or chromic phosphate from the systemic circulation(3,5). Moreover, rabbits receiving a single intravenous injection of a bacterial free meningococcal culture filtrate prior to production of a pneumococcal skin infection show a marked decrease in resistance to the infection (4). The demonstration that phagocytic activity and resistance to experimental infections can be significantly altered by Gram negative bacterial endotoxins suggested that these compounds might alter the immune response. Our study was undertaken to determine the effect of various doses of meningococcal endotoxin on antibody response of rabbits to a single, purified foreign serum protein, crystallized bovine serum albumin.

Materials and methods. Hybrid albino rabbits of both sexes, obtained from a single breeding stock and weighing 2 kilos were used. Rabbits were maintained on diet of Purina rabbit pellets and were free of disease. The endotoxin employed was derived from a strain of meningococcus (44-B) supplied by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York. The method for preparing the toxin has been described in an earlier communication(7). The antigen used was crystallized (BSA) bovine plasma albumin, lot #67009, obtained from Armour and Co. All toxin dilutions and antigen solutions were made up in pyrogen free saline just prior to use. Solutions for injection were prepared so that the final desired dilution of toxin plus the antigen, 15 mg BSA, were contained in total volume of 2 cc. Control animals received 15 mg of BSA in 2 cc volume of pyrogen free saline. Injections were given by way of marginal ear vein. Eleven days after antigenic stimulation, at a time when circulating antibody was presumed to be at its peak level

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TABLE I. Effect of Massive Dose of Meningococcal Endotoxin on Immune Response.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-10 dilution	1652	30		
		1654	80		
		1656	60		
		1651	60		
		1658	36		
		1659	28		
		1661	10		
		1663	60		
			Mean 45 ± 7.62*		
II	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and II				1.259	>.10

* Stand. error of mean.

† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

(8), blood was withdrawn by cardiac puncture and serum separated, quick frozen at –70°C and kept in storage at –30°C until antibody content was determined. The quantitative precipitin technic for determining serum antibody content was a modification of the method described by Heidelberger and Kendall(9). The frozen serum samples were thawed 10 minutes at 37°C, then centrifuged at 3000 rpm at 4°C for one hour. The antibody content was determined at two separate times, a preliminary precipitation using ½ ml portions of serum and final determination using 1 ml of serum. Fresh standard antigen solutions were prepared for each precipitation. Preliminary tests were made to determine antigen concentrations that would precipitate more than ⅓ of the antibody and approach the equivalence point. The serum sample from each animal was precipitated with 4 different concentrations of antigen, incubated at 37°C for 1 hour and placed at 4°C for 7 days. The precipitates were broken up daily by gently swirling contents of each precipitin tube. At end of incubation period the tubes were centrifuged at 2500 rpm for one hour at 4°C, the supernate decanted and precipitates washed with 0.145 molar saline in the cold. This operation was repeated twice. Total nitrogen of precipitate was determined colorimetrically using Heidelberger-MacPherson modification of the Folin-Ciocalteu color reaction(10). Optical density was read at 650 μ on a Coleman Universal Spectrophotometer Model 14. Optical density was converted to nitrogen content by reading a standard calibration curve prepared with a highly purified preparation of human gamma globulin generously supplied by Dr. John T. Edsall, Harvard University. Supernatant analysis for antigen excess was performed in capillary tubes using a hyperimmune anti BSA serum. Capillary tubes were incubated at 37°C for 1 hour, placed at 4°C for 36 hours, then read for presence or absence of a precipitate. Final determinations were performed in identical manner using instead 1 ml serum samples. Antigen solutions for precipitations were made in 0.145 molar nitrogen free sodium chloride solutions. Nitrogen content of standard antigen solution was determined by the micro Kjeldahl method using an all-glass modification of the Markham distillation apparatus. Dilutions of a culture free meningococcal filtrate ranging from 1-10 to 1-10000 were selected to determine their effect on response of rabbits to a standard antigenic stimulation with crystallized bovine serum albumin.

TABLE II. Enhancement of Antibody Production by Meningococcal Endotoxin.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-500 dilution	1013	96		
		1014	163		
		1015	44		
		1016	10		
		1017	50		
		1018	131		
		1019	240		
		1020	35		
			Mean 96 ± 25.74*		
II	Bovine serum albumin, 15 mg Toxin 1-100 dilution	1037	39		
		1038	99		
		1039	139		
		1040	67		
		1042	63		
		1043	49		
		1044	48		
			Mean 70 ± 12.13		
III	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and III				2.378	<.05
II and III				2.95	<.05

* Stand. error of mean.

† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

Results. Effects of Large Dose of Endotoxin on Immune Response. Intravenous injection of 2 cc of the toxin dilution (1-10) produced death within 24 hours after administration in 50% of treated animals. From these effects it was assumed that this dosage of endotoxin is the maximum feasible amount which can be employed to study the effect of meningococcal endotoxin on immune response. As shown in Table I, antibody levels of toxin-treated rabbits, although more variable and slightly higher than those of control rabbits, do not differ significantly from those of the latter group. No evidence of interference with antibody production by this amount of endotoxin was observed. In contrast, as shown in Table II, a smaller amount of endotoxin produced significant enhancement of antibody production.

Optimal Dosage of Meningococcal Endotoxin for Enhancement of Antibody Production. The effect of varying quantities of endotoxin on immune response was investigated. Dosages of endotoxin ranging from large amounts capable of producing death or severe prostration in a high percentage of rabbits (2 cc of a 1-10 dilution) through the range capable of inducing profound circulatory changes, preparation for generalized Shwartzman reaction, and provocation of local Shwartzman reaction, (2 cc of 1-100 and 1-500 dilution) to small dosages capable of inducing a pyrogenic effect but little other evidence of intoxication (2 cc of 1-1000 to 1-10000 dilution) were employed. Results are summarized in Fig. 1. Here it may be seen that while neither the large doses nor the smallest doses of endotoxin used produced significant enhancement of antibody production, increased antibody formation occurred when an intermediate dosage was employed. Optimum dilution of meningococcal endotoxin for this effect lies somewhere between 1-100 and 1-500. This is the amount of toxin

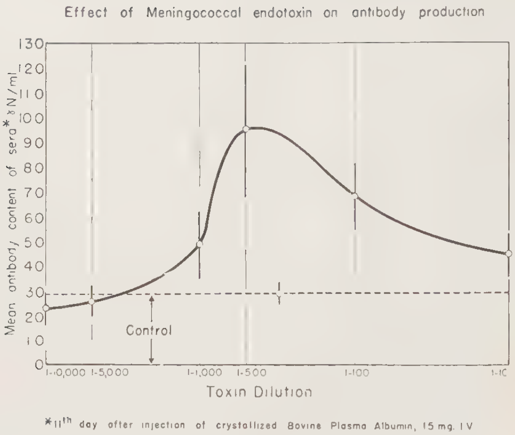

FIG. 1.

which produces profound circulatory changes, preparation for the generalized Shwartzman reaction and provocation of the local Shwartzman reaction in rabbits.

Quantities of toxin capable of producing a vigorous febrile response as well as characteristic alterations of granulocytes (1-5000 to 1-10000) do not act as an adjuvant to antibody production. Slight enhancement of antibody production was noted when 2 cc of the 1-1000 dilution were administered.

Discussion. Increased serum levels of antibody to bovine albumin are produced when a single injection of meningococcal endotoxin is given with it. Maximal enhancement of antibody production was demonstrated with quantities of toxin which are effective in preparing for generalized Shwartzman phenomenon. Massive doses of the toxin did not inhibit immune response as might have been expected from the demonstrated interference of endotoxin with reticuloendothelial function. Instead a slight statistically insignificant enhancement of antibody production was observed. Small quantities of endotoxin which consistently produce a pyrogenic effect and certain other reactions characteristic of this group of substances had no demonstrable effect on immune response of rabbits to the antigen.

Enhancement of immune response with quantities of meningococcal endotoxin employed was an unexpected finding. Cornwell and Good(3) studying the effect of substances such as cortisone, thorotrast and Gram negative bacterial endotoxins on phagocytic activity of reticuloendothelial system found that meningococcal endotoxin, in quantities which this study has shown to enhance antibody production, was most effective in decreasing the rate of clearance of radio colloidal gold from systemic circulation of rats and rabbits. Furthermore, it has been demonstrated that this same quantity of endotoxin results in a marked enhancement of susceptibility of rabbits to experimental infections with pyogenic microorganisms(4). On the basis of these observations it was predicted that a single injection of this dose of endotoxin would result in diminished production of antibody against an unrelated antigen administered simultaneously. Instead of diminishing antibody production the adjuvant effect herein described was observed. The basis for enhancement of antibody production by meningococcal endotoxin is not clear. It is possible that this action is related to the effect of endotoxins on phagocytic capacity of reticuloendothelial system previously mentioned. In support of this concept is the early demonstration by Lewis and Loomis(11) that certain dosages of trypan blue increased production of hemolysin against sheep erythrocytes. Other investigations, however, employing different blockading agents and even different dosages of trypan blue in association with varying immunization procedures, have demonstrated decreased rather than increased antibody production to result from "blockade" of the R. E. system(12,13).

Alterations in distribution of antigen based on changes in circulatory pattern in key organs or even alterations in vascular permeability might be responsible for the enhancement of antibody production herein reported. It seems possible that endotoxins might in some way activate the multipotent reticulum, the differentiation of which appears to be responsible for antibody formation in the rabbit (14). Unfortunately, discussion of the mechanism involved in reactions produced by Gram-negative bacterial endotoxins is hampered by lack of direct information concerning initial site of their action. Indeed, the biologic basis for the toxic properties of these substances has thus far not been elucidated. In general, however, it has been found that reactions produced by endotoxins derived from one group of Gram-negative bacteria are qualitatively indistinguishable from those produced by preparations from other unrelated Gram-negative bacteria(1,2,15). The phenomenon of enhanced antibody production appears to be no exception. In a recent communication, Johnson *et al.*(16) reported a marked enhancement of antibody production against egg albumin when this antigen was given in conjunction with a highly purified lipopolysaccharide antigen (endotoxin) derived from *Salmonella typhosa.*

The recent observations of Stetson(17) that reactions to Gram-negative endotoxins are strikingly similar to reactions produced with tuberculin in specifically sensitized rabbits are provocative and may represent a step toward better understanding of reactions to endotoxins from Gram-negative bacilli. These studies combined with the early demonstration by Lewis and Loomis(18) that guinea pigs infected with tubercle bacilli produce more antibody than do control uninfected animals suggest that perhaps a similar process is responsible for enhancement of antibody production in the two circumstances. Whether tuberculin in appropriately sensitized animals would act as does endotoxin in enhancing production of circulating antibodies is being investigated in our laboratory.

Many agents have been employed to augment production of circulating antibodies. Some of the more common include alum precipitation of antigen, tapioca, staphylococcus toxin and oil-in-water emulsions to which ground killed tubercle bacilli may be added (19,20). The mode of action of these various adjuvants has been generally attributed to production of prolonged inflammatory reactions associated with antigen retention at the site of injection(21-23). Halbert *et al.* (21) reported that with oleacious adjuvants appreciable quantities of antigen remain at the injection site for periods exceeding 22 weeks. However, it seems unlikely that the adjuvant property reported in our studies can be explained on the basis of prolonged retention of antigen at a local depot with consequent slow release and better antigenic stimulation. Rather, it seems likely to us that the ultimate explanation for the adjuvant effect of endotoxin lies in understanding the effect of endotoxin on the cellular system responsible for antibody production. This hypothesis has the advantage of permitting further investigation of the adjuvant action of endotoxin by morphological technics.

Summary. 1. The enhancement of antibody production against crystalline bovine serum albumin by the simultaneous injection of meningococcal endotoxin is reported. 2. An optimum dosage of endotoxin for production of the adjuvant effect is approximately the same as that required to prepare rabbits for the generalized Shwartzman reaction or to provoke the local Shwartzman phenomenon. 3. Failure of inhibition of antibody production by dosages of endotoxin capable of producing delayed uptake of radio active colloidal gold by the reticuloendothelial system is mentioned. 4. Possible mechanisms responsible for the adjuvant phenomenon are discussed.

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Binding of S^{35}-Labeled Sulfate in Serum of Rats as Studied by Filter-Paper Electrophoresis.* (22033)

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Filter paper electrophoresis of blood serum or plasma has been used to study the protein binding of radioactive substances(1-5). Dziewiatkowski(6) recently reported some observations on the association of sulfate sulfur with serum proteins. The present study was undertaken to extend our knowledge of sulfate binding by serum constituents. The results indicate that about 40% of the S^{35}-sulfate administered to Sprague-Dawley rats becomes bound to a substance or substances which migrate to the α_1-globulin region during electrophoresis on filter paper.

Materials and methods. Male Sprague-Dawley rats weighing 300-400 g were injected intravenously with 1200 μc of carrier-free $Na_2S^{35}O_4$. Blood was withdrawn from the heart 24 or 48 hours postinjection with a syringe containing 0.2 ml of mineral oil. The clot was spun down and the serum drawn off with a syringe. The S^{35} activity of the whole serum was determined by counting 100-μl samples in a gas flow proportional counter. Duplicate samples were spread on flat metal planchets and air dried for counting. The serum to be used for electrophoresis was stored at –30°C. Electrophoresis of 25 μl of blood serum placed on 3 by 29 cm strips of Whatman No. 1 chromatography filter paper was carried out in an apparatus similar to that described by Durrum(7). The samples were subjected to electrophoresis for 10 hours in a barbiturate buffer of ionic strength 0.05 and pH 8.6. Best results were obtained when the strips were immersed in the buffer and thoroughly blotted before the sample was applied. The current was maintained at 1.0 ma/strip, and the initial and final potentials were 300 and 150 volts, respectively. Following electrophoresis, the strips were dried at 60°C and developed in tetrabromophenolsulfonphthalein according to the procedure of Durrum(7). The dried and stained strips were scanned with a white light used in conjunction with a Photovolt photometer, and the optical density was recorded at 3-mm intervals. A standard serum electrophoresis pattern was obtained by plotting the optical densities against the distances from the origin. As an indication of the presence of carbohydrates, some of the strips were restained by the periodic acid-Schiff (PAS) routine according to the method of Köiw and Grönwall(8). The radioactivity associated with the serum proteins was determined by cutting the strips into 6 to 24 mm segments. Long or short segments were used where the activity was low or high, respectively. The segments were taped to planchets, and the S^{35} activity was determined in a gas flow proportional counter.

In view of the low S^{35} activity present on the segments of filter paper, it was necessary to

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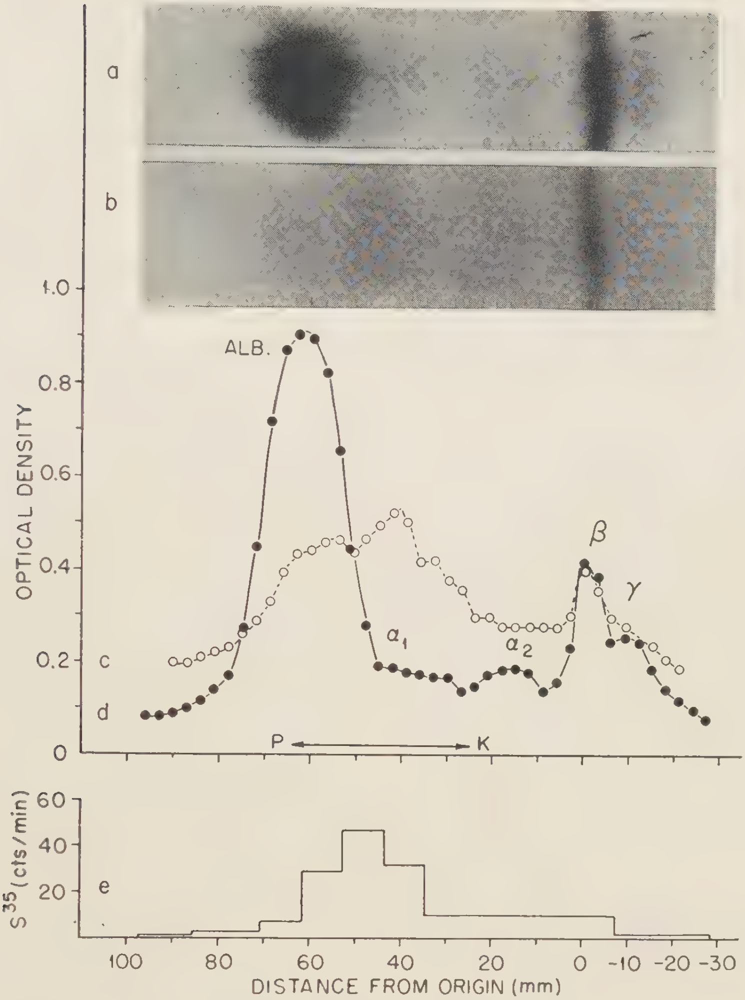

FIG. 1. Electrophoretic pattern of serum proteins as revealed by staining with tetrabromophenolsulfonphthalein (a) and periodic acid-Schiff routine (b). Graph (c) represents the optical densities of (b), and graph (d) depicts the optical densities of (a). Corresponding S^{35}-sulfate activities on the strip are shown in graph (e). S^{35} activity included between points P and K is referred to in the text as the activity peak.

calculate a counting time of sufficient length to insure the significance of the sample counts. A background count was established by averaging the background counts for 31 days. The daily counting periods ranged from 5-20 min., and a single day's value often represented an average of 2 or more such counts. The average value for each day always fell within one estimated standard deviation ($\sqrt{\bar{x}}$) of the mean background for the 31-day period. Since the background did not vary appreciably from day to day, it was considered to be a constant in calculating the sample counting time. It was decided to count each sample long enough so that the gross counts per minute minus 5 times the standard deviation of the gross counts per minute was greater than the background counts per minute. At the maximum sample counting time used, a value of 8 net cts/min. or greater was at least 5 standard deviations from the mean background count per minute. Thus any sample count greater than 8 cts/min. above background was considered to be significant.

TABLE I. Distribution of S^{35} Activity after Electrophoresis on Filter Paper of Serum from Normal Rats Injected Intravenously with $Na_2S^{35}O_4$.

Animal No.	Strip No.	Time post-inj., hr	Whole serum, cts/min./25 µl	Cts/min. in whole strip†	Cts/min. in peak†	% total counts recovered On strip	% total counts recovered In peak	% of recovered counts found in peak
640	91	24	3987	2134	1382	53.5	34.7	64.8
	106	24	3987	2370	1599	59.4	40.1	67.5
	161	24	3987	2270	1510	56.9	37.9	66.5
	159*	24	3987	2426	1596	60.8	40.0	65.8
641	92	24	4468	2384	1671	53.4	37.4	70.1
	100	24	4468	2925	2111	65.5	47.2	72.2
654	143	48	2581	1451	1006	56.2	39.0	69.3
655	144	48	2110	1345	914	63.7	43.3	68.0
645	118	48	1910	1359	844	71.2	44.2	62.1
					Mean:	60.0 ± 1.8	40.5 ± 1.3	67.6 ± 1.0

* This sample was dialyzed for 24 hr in cold against 3 changes of barbiturate buffer prior to electrophoresis.

† Corrected for self-absorption.

To determine what percentage of the whole serum activity remained on all or parts of the strip following electrophoresis, it was necessary to calculate a self-absorption factor. This factor was determined by comparing the activity of aqueous $Na_2S^{35}O_4$ dried on a planchet with the activity of the same solution dried on a disc of Whatman No. 1 filter paper attached to a planchet. The average of several determinations gave a self-absorption factor of 0.359. This factor was used to calculate the aforementioned percentages, but the activity values used to construct graph e in Fig. 1 are the original counts per minute uncorrected for self-absorption.

Results. A representative set of values obtained from the electrophoretic and S^{35} analysis of one serum sample is depicted in Fig. 1. The major part of the S^{35} activity coincided with a region on the strip which contained the α_1-globulin fraction. Occasionally it was observed that this maximum was shifted slightly into the ascending limb of the albumin region, but in no case did this maximum coincide with the albumin peak. The major part of the S^{35}-sulfate also coincided with a region on the strip which stained intensely with the PAS routine.

Calculations from the data for the S^{35} distribution on 9 strips are shown in Table I. It is seen that 60% of the whole serum activity was recovered on the strip, 40% of it being concentrated in a peak,[†] with the remainder distributed over the rest of the area occupied by the serum proteins (Fig. 1).

In one experiment (strip 159, Table I), the serum was dialyzed in the cold (2°C) against 3 portions of the barbiturate buffer during the 24 hours prior to electrophoresis. This procedure did not alter the pattern of distribution or the magnitude of sulfate activity on the strip.

To eliminate the possibility that the observed S^{35}-sulfate distribution on the paper strips resulted from independent migration of free sulfate ions, a serum sample to which $Na_2S^{35}O_4$ had been added *in vitro* was subjected to electrophoresis. The activity in this mixture was twice the amount found in the serum of rats injected with 1200 µc of $Na_2S^{35}O_4$, yet activity analysis of the stained strip showed no S^{35} present on any of the segments.

Discussion. In a paper on the effects of age on sulfate metabolism in the rat, Dziewiatkowski(6) reported that *in vivo* sulfate sulfur "becomes associated with serum proteins, mainly with albumin, and to a lesser extent with α- and β-globulins." The results of the present investigation showed that a major fraction of the serum sulfate is combined with a substance having the electrophoretic mobility of an α_1-globulin.

[†] The S^{35} peak includes the activity present between the maximum point of the albumin curve and the low point anodal to the α_2-globulin region, *i.e.*, between points P and K in Fig. 1.

Sulfur is known to be present in 3 forms in the serum: one fraction is present as the sulfur in amino acids; another fraction is in the form of inorganic sulfate ion; and a third fraction is represented by several sulfuric acid esters, *e.g.*, heparin and indoxyl sulfuric acid. It has been shown by Tarver and Schmidt(9), and Boström and Jorpes(10) that administered S^{35}-sulfate does not become incorporated into the sulfur-containing amino acids of the rat, or becomes incorporated in negligible amounts (11). Our results, therefore, cannot be explained in terms of -S-S- or -SH groups.

The absence of activity on the paper strip following electrophoresis of serum mixed with S^{35}-sulfate *in vitro* confirms the recent report of Dziewiatkowski(6) that binding does not occur under *in vitro* conditions. In addition it indicates that the S^{35} in the α_1-globulin region did not result from the presence of free sulfate ions. Furthermore, the removal of free sulfate ions by dialysis of S^{35}-sulfate-labeled serum prior to electrophoresis resulted in no change in the amount of activity remaining on the strip after electrophoresis. These findings do not eliminate the possibility of a salt linkage between the sulfate ion and a protein, but the following preliminary experiments in this laboratory do not support such an hypothesis. After dialysis of S^{35}-sulfate-labeled plasma against a buffer of pH 12.0, 38% of the whole activity remained in the plasma, essentially the same amount (40%) as was found in the α_1-globulin region following electrophoresis of undialysed plasma at pH 8.6. At pH 12.0, few, if any, positively charged groups of a protein would be available for anion binding through a salt linkage. Even at pH 8.6, it is expected that the number of available groups of a protein capable of binding $SO_4^=$ would be small. In addition, anion binding would have to be quite strong to overcome the attraction of the sulfate ion to the anode during electrophoresis at pH 8.6. Although anion binding to a protein through a salt linkage might explain the present results, it seems more likely that the S^{35}-sulfate activity peak reflects the presence of sulfuric acid esters.

Proteins combined with sulfomucopolysaccharides such as chondroitin sulfate occur in many tissues. Protein-carbohydrate complexes such as the mucoprotein described by Winzler *et al.*(12) and an α_1-glycoprotein crystallized by Schmid(13) have been found in plasma. These 2 substances, isolated from human plasma and apparently identical, are acid although the composition of the acid groups of the carbohydrate moiety is unresolved(14). Schmid reported that his α_1-glycoprotein did not contain a sulfuric acid ester. Winzler *et al.*(12), and Weimer *et al.*(15) found, however, that after mild hydrolysis of their mucoprotein preparations a precipitate was formed by the addition of barium chloride to the hydrolyzate, suggesting that labile sulfuric acid groups had been removed from the mucoprotein. Winzler's mucoprotein has the electrophoretic mobility of an α_1-globulin at pH 8.4 (16), and contains approximately 30% carbohydrate(15). In the present studies it was observed that the S^{35}-sulfate activity peak corresponded to an area on the strip which contained PAS-positive constituents. Since the S^{35}-sulfate activity peak and the PAS-positive region appear to coincide electrophoretically with the mucoprotein described by Mehl *et al.*(16), it is possible that the carbohydrate moiety of this mucoprotein is esterified with sulfuric acid. A similar interpretation was previously suggested by Mehl *et al.* (16) to account for the observation of Winzler and Burk(17) that sulfur in excess of that required by cystine and methionine was present in a mucoprotein preparation from rat blood. With the use of human blood, Weimer *et al.* (15) made the same observation.

Summary. The binding of S^{35}-labeled sulfate in rat serum has been investigated by filter paper electrophoresis. About 40% of the whole serum S^{35}-sulfate activity was found to be combined with a serum constituent having the electrophoretic mobility of an α_1-globulin. Although the chemical identity of the labeled substance has not yet been established, the results of these experiments suggest that it may be a protein with a sulfonated carbohydrate prosthetic group.

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Effect of Some Centrally Acting Drugs on Compulsive Circling. (22034)

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The group of signs and symptoms observed, following a unilateral intracarotid injection of di-isopropyl fluorophosphate (DFP) into rabbits, has been called the adversive syndrome (1) and the latter has been attributed to the anticholinesterase action of DFP(1-5). As a result of DFP administration, an asymmetric distribution of cholinesterase (ChE) activity is produced in the frontal cortex and caudate nucleus. The enzymatic activity is profoundly depressed on the injected side of the brain whereas the opposite side is relatively unaffected. This is especially marked in the right caudate nucleus which retains only 4% of its normal ChE activity. This inhibition permits asymmetric accumulation of acetylcholine (ACh) in the brain(6) which in turn may be directly responsible for the observed effects. It has been established in cats and monkeys that the adversive syndrome is the result of a biochemical lesion of the central nervous system(7). Although it is possible that peripheral receptors are more important in rabbits than in cats(7), many factors also point to a central mechanism for the production of the adversive syndrome in the former animal. First, the direct application of DFP to the peripheral vestibular receptors elicits no behavioral change in rabbits(8). Secondly, central stimulants can modify the forced circling behavior(5). Thirdly, a characteristic asymmetric brain cholinesterase pattern is found in all animals exhibiting the adversive syndrome, and this pattern is absent when compulsive movements are not observed (3,9,10). Finally a temporary or permanent reversal in the direction of the forced movements usually follows a minor convulsive seizure(2). The factors mentioned above appear to implicate the central nervous system as the primary site for the production of compulsive circling in rabbits. Previous work(5) showed that the adversive syndrome can be corrected by intravenous injections of small amounts of atropine sulfate after which the animal exhibits its normal mode of progression. When large doses (i.v.) of atropine are given the forced circling is reestablished, however, the animal turns in the opposite direction, toward the injected side.

In the present study a further analysis of

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TABLE I. Effect of Atropine-Like Drugs on the Adversive Syndrome (Dose of Drug, mg/kg).

Drug	Reversal after atropine*			Reversal without atropine		
	No. of exp.	Mean	Range	No. of exp.	Mean	Range
Scopolamine	5	.04	.02 -.08	4	.09	.06 -.12
Artane	8	.05	.02 -.10	6	.07	.02 -.12
Panparnit	6	.2	.1 -.3	5	.2	.1 -.4
WIN 2299	5	.015	.005-.02	5	.013	.005-.025

* Adversive syndrome was corrected by intravenous administration of atropine before experimental drug was injected.

this reversal is based on the ability of a variety of centrally acting drugs to produce this phenomenon. It is worth noting that the preparation to be described may be used as a bioassay for the atropine-like properties of drugs which act on the central nervous system. This bioassay is valuable because it establishes the drug action in terms of a behavioral response, namely the reversal in direction of the compulsive circling, in contrast to other assays which measure the drug effect on a single isolated organ or tissue.

Method. The adversive syndrome was produced in albino rabbits (approximately 2 kg) as described previously(5). The following drugs were used: atropine sulfate, scopolamine (hyoscine hydrobromide), Artane (trihexyphenidyl), Panparnit (diethyl aminoethyl-1-phenylcyclopentane-1-carboxylate hydrochloride), WIN 2299 (2-diethyl aminoethyl cyclopentyl-(2-thienyl) glycolate hydrochloride), Benadryl hydrochloride (diphenydramine hydrochloride), Diparcol (diethylaminoethyl-N-dibenzoparathiazine hydrochloride), LSD (d-Lysergic acid diethylamide), Reserpine (an alkaloid from Rauwolfia serpentina Benth), and Lergigan [N (2-dimethyl amino-2 methylethyl) phenothiazine]. All drug injections were made into a marginal ear vein. The *average values* (mg/kg) for a single intravenous injection of each drug were: atropine 0.2, scopolamine 0.02, Artane 0.02, Panparnit 0.1, WIN 2299 0.005, Benadryl 0.2, Diparcol 1.0, LSD 0.01, Reserpine 0.5, and Lergigan 0.2. Following each intravenous injection of a test drug, the rabbit was allowed to move freely on a rubber mat. The injections were continued at 2 to 6 minute intervals until the direction of the forced circling was reversed from that seen immediately after the administration of DFP. This reversal of forced circling was used as the end point in establishing the effect of the drug on the CNS. In those cases where no reversal of the forced movements was noted the injections were continued until the animals appeared to be in respiratory difficulty, in convulsions, or asleep. The last effect was noted only after Reserpine. In some cases atropine was administered prior to the test drug. This was done to obtain information on the synergism of atropine and the test drugs.

Results. The data obtained with atropine-like drugs are shown in Table I. In these experiments the amount of drug necessary to produce a reversal of the adversive syndrome was established in 2 groups of animals: 1) following atropine correction, and 2) without prior atropine administration.

The direction of the forced circling was reversed by a mean dose of 0.04 mg/kg of scopolamine when atropine was first injected in amounts sufficient to correct forced circling. However, 0.09 mg/kg of this drug was needed without atropine. The difference in these means was significant to beyond the 1% level when tested by the Student "t" test. Therefore, the pharmacological effects of scopolamine and atropine appear to be additive in our preparation.

The other 3 atropine-like drugs included in Table I, Artane, Panparnit and WIN 2299, required approximately the same dose of drug to produce a reversal in the direction of the forced circling whether or not atropine was first administered. In contrast to the results obtained with scopolamine, a synergism was not noted when these 3 drugs were used with atropine.

The effect on the adversive syndrome of some other drugs known to act on the CNS are presented in Table II. In general, these

TABLE II. Effect on Adversive Syndrome of Some Drugs Known to Act on the CNS (Dose of Drug, mg/kg).

	No reversal			Reversal		
Drug	No. of exp.	Mean	Range	No. of exp.	Mean	Range
Lergigan	3	2.7	1.2 - 3.5	5	1.8	1.2-2.8
Benadryl	8	3.0	1.0 - 6.6	2	.6	
Reserpine	5	4.6	3.0 - 6.0			
Diparcol	3	9.2	4.5 -12.0	1	5.0	
LSD	5	.05	.015- .09	1	.02	

drugs do not have a strong atropine-like action and were therefore administered to the rabbits without prior atropine treatment. Of the five drugs tested, Lergigan was best able to reverse the direction of the forced circling. It should be noted that a reversal was observed in 5 of the 8 animals studied.

Benadryl, a potent anti-histaminic reversed the direction of forced circling in only 2 of 10 animals tested. It was therefore far inferior to Lergigan in its ability to change the direction of forced circling. *Reserpine* does not affect the forced circling. Each of the 5 rabbits given this drug appeared to fall asleep with a scoliosis away from the injected side. A gentle tapping on the spine aroused the animal and it then circled about several times before resuming its sleeping posture. *Diparcol* reversed the involuntary movements in only one of the 4 rabbits tested. However, the mean dose needed was much larger than that of the other drugs listed in Table II. *LSD* was similar to Diparcol in its lack of a marked effect on forced circling. The direction of the compulsive movement was reversed in only one of the animals studied. In general, it appeared to be quite toxic when administered following the intracarotid injection of DFP.

Additional observations were made on ability of the listed drugs in Table II to correct the adversive syndrome. The corrected animal exhibits a normal behavioral pattern and may move in any direction. In a given animal the correction always occurs with a smaller dose of drug than that needed for reversal. In the present experiments this phenomenon was observed only with Lergigan and Benadryl. In the case of Benadryl 4 of the 10 rabbits were corrected, whereas 7 of 8 other rabbits studied were corrected by Lergigan.

Discussion. It has been previously reported (5) that small amounts of atropine correct the forced circling produced in rabbits by the unilateral intracarotid injection of DFP. This effect was referred to the power of atropine to block the action of accumulated ACh. On the other hand, large doses of atropine restored forced circling, but in the opposite direction. Since several CNS stimulants can also evoke a reversal in forced circling, it was suggested that this latter effect of atropine was due to a stimulant action. The fact that stimulation of the cortex may be important in producing a reversal is given further support from the observation that a mild convulsive seizure may be followed by such a change in the direction of the forced movements.

Of the drugs used in our study, the reversal in forced circling was most easily obtained with the atropine-like ones. However, it is apparent that the syndrome can be produced by a variety of drugs. These may be roughly divided into two categories: a) the CNS stimulants and b) the atropine-like drugs.

The suprabulbar vestibular system is complicated and the mechanism by which it is affected in the present preparation is unknown. However, two possible hypotheses may be suggested to explain the production of the reversed adversive syndrome: (a) the more nearly normal enzyme content of the left cortex may allow the intravenously injected drug selectively to stimulate that side more than the enzymatically depressed right side, thereby resulting in a reversal in the direction of forced circling, or (b) the production of convulsive activity on both sides of the cortex is followed by a bilateral depression in cortical activity. Both of these mechanisms would "neutralize" the original asymmetric activity of the cortex which follows the unilateral intracarotid injection of DFP. In the first case

(a) the reversal is then attributed to a stimulation of the left cortex by the injected drug and in the second (b) to the biochemical lesion in the right caudate nucleus which appears to be so severe as to preclude restoration of normal function by the administration of atropine or other drugs. It has been shown (11) that ablation of the right caudate nucleus (with or without destruction of the cerebral cortex) results in circling toward the right. The brain areas involved in the production of the adversive syndrome as well as the mechanisms by which drugs affect this system are at present the subject of a series of investigations in our laboratory.

The usefulness of the present method as a bioassay is demonstrated by the results obtained with the experimental drug WIN 2299. It is shown to be similar to Artane, Panparnit and Lergigan in its effect on forced circling. These drugs play an important role in the treatment of Parkinsonism. Clinical trials with WIN 2299 would seem to be profitable from our present results.

Summary. The intravenous injection of scopolamine, Artane, Panparnit, Lergigan or WIN 2299 into rabbits exhibiting the adversive syndrome evoke a reversal in the direction of the compulsive circling. Of the drugs mentioned, only scopolamine acts synergistically with atropine in causing this reversal. The usefulness of the procedure outlined as a bioassay for atropine-like drugs is discussed and two possible mechanisms by which the drugs studied produce this reversal are mentioned.

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Practicability of Shipping HeLa Cell Suspensions for Use in Color Test For Poliomyelitis Antibodies.* (22035)

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The continuous cultivation *in vitro* of the human cancer cell (HeLa) by Gey and coworkers(1), and the important finding that these cells showed cytopathic effects in the presence of poliomyelitis virus(2), has made possible the use of many tests for poliomyelitis investigations without maintaining a monkey colony. Small laboratories are now better able to make such investigations providing ready access to a good supply of HeLa cells is available. The authors have previously reported their observations on the quantitative titration of 2000 human sera against 3 prototype strains of poliomyelitis virus employing as indicator system color changes of medium in tubes containing suspended HeLa cells(3).

This report concerns the practicability of employing suspensions of HeLa cells shipped to a laboratory for use in carrying out the same method.† The observations were undertaken in order to: (a) learn whether a small

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† Suspensions were obtained from George Washington Carver Fn., Tuskegee Institute, Ala., and may be procured from Dr. R. W. Brown there.

laboratory without special equipment could utilize this technic, (b) circumvent shipment of older cultures with heavy cellular growth since these may fall off during shipment, (c) reduce the cost of shipping numerous bottles, and (d) determine the practicability of shipping suspensions of cells for the initial source of a colony. It was observed by Youngner (4,5) that trypsinized monkey kidney cell suspensions could be shipped at the temperature of melting ice and were usable after a day or more. More recently, Melnick and associates (6), have described experiments in which monkey cell suspensions were prepared in India and flown to the United States.

Method. HeLa cell suspensions were prepared at Tuskegee Institute from young and old cultures which were fed shortly before preparation and shipment of suspensions. Cells were cultured in 250- to 500-ml bottles and dispersed with trypsin according to the method described by Scherer, Syverton, and Gey(2). For shipment the dispersed cells, 2-2.5 million per ml, were suspended in a medium of 40% human serum and 60% Hanks' solution. Penicillin and streptomycin were added to respective concentrations of 75 units and 75 μg per ml. The volume shipped was 100 to 125 ml in a glass container whose volume was 125 ml. The container was shipped at room temperature. Those suspensions made up on the afternoon of day 0 were received in our laboratory by noon of the following day (day 1). The suspension was stored at refrigerator temperature (4°C) and used usually on day 2 or day 3. The cell count was checked in a hemacytometer upon receipt of suspension and was within a reasonable range of deviation from the number reported shipped (minus 20%). On removal from the refrigerator the supernatant fluid was removed by suction and the cells washed in 40 ml of medium 199 containing 5% monkey serum and additional glucose(3). The low speed centrifugation for 10 minutes at 500 rpm was followed by removal of the supernate and cell washing for a total of 3 times. Finally, the cells were suspended in medium 199 containing 5% monkey serum with the additional glucose, adjusted so that each ml contained 200000 HeLa cells. Resulting suspension was then used in the colorimetric test(3).

TABLE I. Reproducibility of Serum Antibody Titers. HeLa cell bottle cultures vs. HeLa cell suspensions.

	Type I	Type II	Type III
Titers same*	120	112	103
Titers discrepant			
± 1 tube	50	57	62
± 2 tubes	7	15	14
± 3 or more tubes	2	1	6
Total No.	179	185	185
% reproducible (± 1 tube)	95%	91%	89%

* Highest dilution 1:1024.

Results. The technic outlined above has been employed to determine the antibody titers of 185 sera which have been titrated 7 months previously with cells shipped as bottle cultures, and trypsinized just before use to make suspensions for the test(3). Both tests were performed using a single tube per dilution per virus type with the following serum dilutions: 1:4, 8, 16, 64, 256, 1024. Table I includes the number of agreements and discrepancies which appeared when titers were compared. These titers were obtained in scattered tests, 13 different combinations of tests. Replicate titration results differed by no more than ±1 tube whether employing 2-fold or 4-fold dilution series. Sera which were recorded as agreeing by both methods ranged in titer from less than 1:4 to greater than 1:1024. Sera with titers recorded as greater than 1:1024 were not titrated further. Of 179 sera titrated against Type I virus, 120 were in agreement and 50 differed by ±1 tube (95% reproducible). A similar situation obtains with Type II and Type III antibody titrations with a reproducibility of 91% and 89%, respectively.

Forty-six shipments of cell suspensions were utilized in the titration of 4000 sera against Types I, II, and III poliomyelitis viruses. Control monkey sera of known titer, virus pools of known tissue culture infectivity and human sera of known antibody content‡ have, when titrated by this method, yielded results in close agreement with known titers. Technically satisfactory titrations have been accomplished using cells sent from a central source and treated according to the method described above.

Summary. It has been found feasible to ship large numbers of suspended HeLa cells packed in a small container. Neutralization titers of 185 sera tested with 2 different types of shipped HeLa cells have yielded reproducible results. Under the conditions described the neutralizing capacity of 4000 serums has been tested against 3 prototype strains of poliomyelitis virus in a color test.

The authors express deep appreciation to Dr. R. W. Brown for his thoughtful cooperation in planning this study.

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Plasma Iodine^{131} Levels as Diagnostic Tests for Lethal X-Radiation Exposure in Rats. (22036)

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At present there is no single, simple, diagnostic laboratory test for lethal exposure of man or experimental animals to ionizing radiation. In reviews of clinical diagnosis and treatment of radiation injury, Dunham *et al.*(1) and Cronkite(2) have pointed out that determination of the sequence of occurrence of various signs and symptoms is the principal method for establishing the severity of radiation exposure in man. Of various clinical laboratory tests, the white blood cell count appears to be of most value for prognosis(1,2). Relatively few diagnostic tests have been reported for experimental animals. Rosenthal(3) found an opalescence in the serum of rabbits exposed to lethal doses of x-rays and reported a correlation with lethality. Entenman *et al.* (4) confirmed this finding in rabbits but were unable to demonstrate it in other species. These workers also determined plasma phospholipid phosphorus (PLP) levels in a number of species of experimental animals and concluded that though they might be of value for prognosis of mortality in the dog, the PLP level did not serve as a good indicator of radiation injury "since in all species lethal doses of irradiation (LD_{50} to LD_{100}) are required to produce appreciable changes." Hayes and Hewitt(5) separated the plasma lipids of irradiated rabbits into various "classes" by ultracentrifugation and found an excellent correlation between increases in certain classes and mortality. Their method, however, has the disadvantage of complexity and is probably not adaptable to routine use. The uptake of radioactive iron by red blood cells of irradiated animals is known to be well correlated with radiation dose(6-9). The apparent disadvantage to this method is, that while it is sensitive to relatively low dose ranges, the relationship does not hold well in the lethal ranges since maximum depression occurs at less than lethal levels. Gershon-Cohen and Hermel(10) have recently reinvestigated the very early changes in the white blood cell count of irradiated rats and have concluded that such information may be of value in prognosis.

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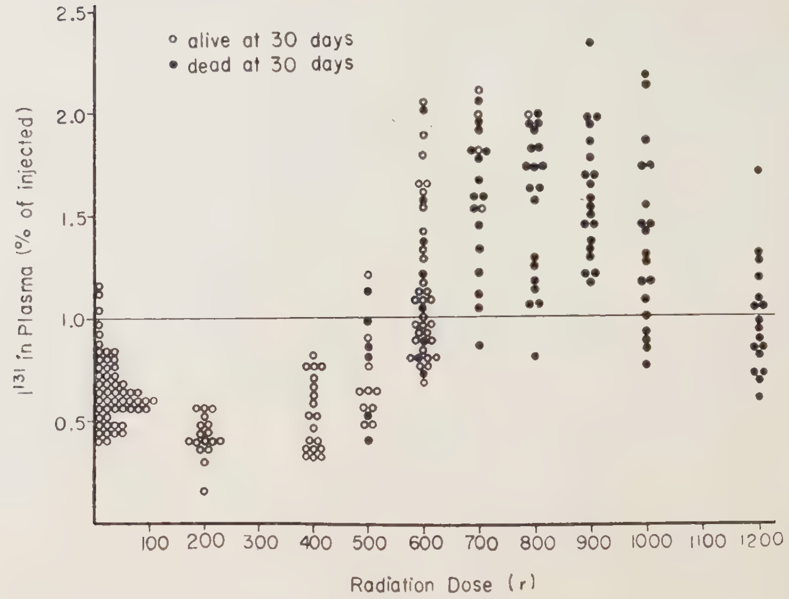

FIG. 1. Effect of various doses of x-radiation on iodine^{131} levels in the plasma of rats.

In the first of a series of projected studies on the distribution of fission products in irradiated rats it was noted that there appeared to be a relationship between plasma iodine^{131} level and the subsequent fate of the animal. This relationship was studied in more detail and is the subject of the present report.

Methods. A total of 242 adult male Sprague-Dawley rats was used. Since it was desirable to use as heterogeneous a group as possible, the rats selected weighed from 200 to 550 g and varied in age from approximately 3 to 18 months. They were housed 3 to a cage and given water and Purina Laboratory chow *ad libitum*. X-rays were delivered from a General Electric Maxitron unit using the following factors: 250 KVP, 30 ma, added filters of 1.0 mm Al and 0.5 mm Cu, TSD 50 cm, dose rate—115 r/min. The rats were exposed 3 at a time in a shallow lucite cage. The iodine^{131}, obtained from the Oak Ridge National Laboratory, was diluted with Krebs-Ringer phosphate buffer (pH 7) to a concentration of 5 μc/ml. Each rat received 1.0 of the solution intraperitoneally 48 hours following x-ray exposure. At that time suitable aliquots were set aside for counting at the same time that the plasma iodine determinations were made. Twenty-four hours after the injection of iodine (72 hours after irradiation), the animals were lightly anesthetized with ether and one ml of blood was removed into a heparin-wet syringe by cardiac puncture. The rats were allowed to recover, returned to their cages, and their survival was followed for 30 days. Autopsies were performed on animals dying the first few days following this procedure and any which showed evidence of hemorrhage from the puncture were excluded from the final tabulation of results. Blood obtained by cardiac puncture was placed in conical centrifuge tubes and the red cells were spun down. A 0.2-ml aliquot of plasma was then placed in a planchet containing 0.4 ml of 0.375 M NaOH and one drop of phenolphthalein solution. The solution was slowly evaporated to dryness under an infrared lamp and the iodine activity determined by beta counting under a thin-window Geiger-Muller tube connected to a conventional scaler. The iodine^{131} activity in the total plasma volume was calculated as per cent of the injected dose by the formula:

$$\frac{\dfrac{(\text{Counts/min. in 0.2 ml plasma})}{(\text{body wt in g})(14.725)}}{(\text{Counts/min. in 1 ml of inj. sol.})} = \% \text{ of inj. dose in plasma.}$$

The factor 14.725 was obtained on the basis of the following considerations: (a) It was

TABLE I. Accuracy of Test in Prognosis at Various Doses of Radiation.

Radiation dose	No. rats	No. dead (30 days)	Positive values*				Negative values*				Combined accuracy, %
			Total	Correct	False	Accuracy, %	Total	Correct	False	Accuracy, %	
0	55	0	3	0	3	0	52	52	0	100	95
200	17	0	0	0	0	—	17	17	0	100	100
400	19	0	0	0	0	—	19	19	0	100	100
500	16	6	2	1	1	50	14	9	5	64	62
600	39	8	21	5	16	24	18	15	3	83	46
700	20	16	19	15	4	79	1	0	1	0	75
800	20	19	19	18	1	95	1	0	1	0	90
900	20	20	20	20	0	100	0	0	0	—	100
1000	19	19	14	14	0	100	5	0	5	0	74
1200	17	17	7	7	0	100	10	0	10	0	41

* Plasma I^{131} values in excess of 1% were considered ''positive.'' Values of 1% or less were considered ''negative.''

assumed that there were 5.89 ml of blood/100 g body weight(8); (b) The hematocrit was assumed to be 0.50; (c) The counts/min. in the 0.2-ml sample of plasma were multiplied by 5 to convert to c/m/ml; (d) The entire equation was multiplied by 100 to convert to per cent,

Thus: $\frac{5.89}{100} \times 0.50 \times 5 \times 100 = 14.725.$

Results. Plasma levels of I^{131} (as per cent of injected dose) at various radiation doses are shown in Fig. 1. As radiation doses were increased in the range of 0 to about 900 r an increasing proportion of the rats showed relatively high levels of I^{131} in the plasma. At higher dose levels there appeared to be a reversal of the effect and the incidence of high values decreased.

Rats of the strain used in this study rarely die from radiation exposures of less than 500 r. At doses of 500 to 900 r, the incidence of lethality increases and at doses in excess of 900 r the incidence is nearly always 100%. The dose level of 500 r, then, can be taken as the point below which survival is reasonably certain and above which death may occur. From Fig. 1 it is apparent that I^{131} values of 1% or less were found in nearly all the control rats and rats exposed to less than 500 r. In the range of 500 to 900 r an increasingly greater percentage of the animals showed values in excess of 1%. For this reason, the 1% level was arbitrarily chosen to divide the results into "positive" (high) and "negative" (low) values. It was then assumed that positive values were prognostic of death and that negative values were prognostic of survival and the results were evaluated for accuracy as a possible diagnostic test. Table I lists the incidence of positive tests and lethality at various dose levels. In addition a tabulation of correct and incorrect predictions based on positive and negative I^{131} tests at each dose has been included. In this tabulation, death in a rat showing a positive value was considered a correct prediction as was survival in a rat showing a negative value. Death in rats showing negative tests or survival in rats showing positive tests were listed as false negatives or false positives, respectively. Percentage accuracy of positive and negative tests are shown separately and in the final column the over-all accuracy at each dose level is shown.

The test was not accurate at radiation doses in excess of 900 r, since a tendency to reversal of the effect occurred at these levels. This finding indicated that the test was valid over a restricted dose range and the doses in excess of 900 r exceeded this range. Accuracies at 1000 and 1200 r were computed in the same manner as for lower doses and, in the interest of completeness, are shown in Table I.

Discussion. Cronkite(2) has pointed out that for purposes of triage, a population exposed to ionizing radiation may be divided into the following groups: a. Sublethal exposure—recovery probable; b. Lethal exposure—recovery possible; c. Supralethal exposure—recovery improbable.

For purposes of evaluating the accuracy of the present test in dividing the sample rat population into these 3 groups, the population was broken up as follows: a. Sublethal exposure—0 to 400 r; b. Lethal exposure—500 to 900 r; c. Supralethal exposure—over 900 r.

The assignment of the dosage ranges to these 3 groups was made arbitrarily based on the following considerations: Rats exposed to 0 to 400 r usually survive. In the range of 500 to 900 r some, but not all, of the rats usually die. With present methods of treatment of radiation injury, it is possible to increase survival in this range. Doses in excess of 900 r are uniformly fatal and survival is little influenced by present methods of therapy.

There was a total of 91 rats in the sublethal exposure group, all of which survived. Since 3 rats showed false positive values, the overall accuracy in this range was 97%. Tests were run on 115 rats exposed in the lethal (500 to 900 r) range. Twenty-two rats showed false positive and 10 showed false negative values for an accuracy of 72%. The principal source of error in this dose range was in false predictions of death (false positives). Since such errors would not be serious if the test had been used as an indication for instituting therapy, it may be permissible to ignore them and consider only the false negatives as errors. In this case, the accuracy in the lethal range was 91%.

Only 2 doses in excess of 900 r were employed, namely, 1000 to 1200 r. These doses may properly be considered supralethal. Of the 36 rats tested, 21 showed positive values and 15 showed negative values. All the rats died. The accuracy in predicting death, then, was only 58%. The increased incidence of negative values in this dose range may be of value in triage. The diagnosis of severe radiation illness was obvious from the severe weight loss, diarrhea, ruffled fur, etc. With present methods of treatment of radiation illness, it would undoubtedly have been impossible to prevent death in these animals. The combined clinical impression of severe damage and a negative I^{131} test may facilitate the diagnosis of supralethal exposure. Further investigation of these and higher dosage levels is, of course, required.

If the values for accuracy at doses in excess of 900 r are excluded, then the over-all accuracy of the test in the 0 to 900 r range was 83 to 95%, depending on whether false-positive values are acceptable.

It must be emphasized that the test as employed in the present study was entirely empirical. The mechanism by which the plasma levels of I^{131} were increased is not known. Refinements in technic or the use of different time intervals might increase the accuracy. At present, it may be stated that under the conditions of the experiment it was possible to diagnose exposure to sublethal and lethal doses of x-radiation with fair accuracy over a restricted dose range.

Summary. By determining plasma levels of trace amounts of radio-iodine in rats 24 hours after injection and 72 hours after x-irradiation with 0 to 900 r, it was possible to divide the animals into sublethal and lethal exposure groups with an accuracy of 83 to 95%, depending on whether a false diagnosis of lethal exposure is acceptable. There appeared to be a reversal of the effect on I^{131} level at doses in excess of 900 r, which might be of value in triage. At these dose levels, the diagnosis of severe radiation injury was obvious from the appearance of the animals and the combined clinical impression of severe damage and a negative I^{131} test might facilitate the diagnosis of supralethal exposure.

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Bacterial Allergy Increases Susceptibility to Influenza Virus in Mice.*
(22037)

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We found previously that the vaccination of mice with *H. pertussis* vaccine increased their susceptibility to infection with several unrelated species of Gram-negative bacteria; such mice succumbed from a smaller number of live bacteria than normal mice. These results stimulated our interest in determining whether sensitization of mice with *H. pertussis* vaccine could make them more susceptible to virus. Experiments on this subject so far performed with influenza virus are described below.

Materials and methods. We used the CFW strain female mice of different ages: young mature females weighing about 22 g and weanlings weighing 10-12 g. Influenza virus A, strain WS, harvested in chick embryo allantoic fluid, with a hemagglutinin titer of 1:1280 was used for the intranasal inoculation of the mice. Sensitization was performed by the intraperitoneal injection of 15 billion cells of commercial *H. pertussis* vaccine. Experiments with sensitized mice were started 5-7 days after vaccination with *H. pertussis* and mice were observed for 2 weeks after the administration of the virus.

Results. Immunization with *H. pertussis* antigen considerably reduced the resistance to virus in mice of different ages. Normal and sensitized mice died within 5-10 days after an inoculation with the virus; however, the lethal dose of the virus for sensitized mice was much smaller than for normal mice of the same age group (Table I).

The young weanling mice which had been inoculated with the *H. pertussis* vaccine had become hypersensitive and could be shocked with the homologous antigen. The shocking dose of *H. pertussis* nucleoprotein had no effect on the normal, but killed 3 out of 6 of the sensitized weanlings. Adult mice injected with the same number of *H. pertussis* cells became sensitized to a higher degree; the same shocking dose of nucleoprotein produced up to 80-90% of anaphylactic deaths among the sensitized adult mice.

Four control groups of sensitized mice were used to demonstrate the mode of action of the influenza virus (Table II). When the influenza virus was inactivated and inoculated intranasally into sensitized mice it did not kill them, in contrast to the active virus, which in a dilution of 1:10000 given intranasally killed most of the sensitized mice. However, active virus, even undiluted, was well tolerated by sensitized adult mice if it was injected intraperitoneally.

Special studies were performed on the effect of sensitization by *H. pertussis* on the protection of mice by specific anti-viral antibodies. Immune serum was prepared by the injection of the same WS strain of influenza virus into rabbits. The immune serum had an anti-hemagglutinin titer of 1:5000. We studied the effect of the antibodies on the inactivation of the virus *in vitro* and *in vivo*. In

TABLE I. Susceptibility to Influenza Virus of Normal and Sensitized Mice of Different Ages.

Virus given .05 ml intranasally in dil.	Adult mice			Weanling mice		
	Normal	Sensitized	P value	Normal	Sensitized	P value
1: 1000	15/30	18/23	.10 > P >.05	31/35		
1: 10000	1/17	13/17	P <.001	16/22		
1:100000				6/19	15/18	P <.01

No. of dead mice in numerator; total No. of mice in denominator.

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The author wishes to thank Dr. J. F. McCrea for his help in the production and titration of virus and antiserum.

TABLE II. Different Pretreatments of Influenza Virus and Different Routes of Administration to Sensitized Adult Mice.

Material used for inoculum	Route of admin. and dilution	Results
Virus inactivated 30 min. at 56°C	Intranasally, .05 ml, dil. 1:100	0/ 6
Virus inactivated by incubation with 1% formalin at room temp., 24 hr	*Idem*	0/ 6
Allantoic fluid from non-infected eggs, same age as those used for harvest of virus	Intranasally, .05 ml, undil.	0/ 5
Active virus	Intraper., .2 ml, undil.	0/ 6
Idem	Intranasally, .05 ml, dil. 1:10000	13/17

the first series of experiments the virus in a dilution of 1:10 was mixed with an equal volume of antiserum diluted 1:10. After 30 minutes incubation at room temperature, .05 ml of the mixture was administered intranasally to each mouse. As a control, virus diluted in saline 1:20 was given intranasally in .05 ml volume. Normal and sensitized mice were used for this test and, since the results of both groups were the same, the data were combined. Virus inactivated with antiserum *in vitro* was not infectious for either normal or sensitized mice.

Different results were obtained in experiments in which mice received antiviral serum intraperitoneally and virus intranasally. In this case the protection of sensitized mice was very poor in comparison to normal mice. In one experiment some mice received immune serum undiluted; others in dilutions of 1:10 and 1:100; since protection with serum undiluted and diluted 1:10 was about the same in each group of normal and sensitized mice, the data were combined. Half of the mice in our tests were sensitized by *H. pertussis* vaccine as described above. In these experiments mice started to die 5-10 days after the injection of virus and were kept under observation for 2 weeks (Table III). An autopsy of the dead mice revealed lesions in the lungs typical of an influenza virus infection.

Discussion. The striking effect of the *H. pertussis* vaccine on the sensitization of mice and guinea pigs was described by the author and his collaborator in 1947(1). The development of increased sensitivity to histamine after vaccination with *H. pertussis* was observed by us in 1948(2), and since that time various studies of this phenomenon have been performed(3-5,8). In 1953 we observed(6) that vaccination with *H. pertussis,* which produced an immunity to the homologous organism, at the same time lowered the resistance of the mice to non-related infections with different Gram-negative bacteria and increased their susceptibility to *Shigella* endotoxin(7).

(1) Vaccination of mice of different ages with the *H. pertussis* antigen increased their sensitivity to the influenza virus (Tables I and III). The increase in the death rate in vaccinated mice in comparison to the normal animals of the same group could not be attributed to anaphylactic shock, but rather to the lowering of their resistance to an influenza virus infection. In vaccinated mice, as in normal animals, death occurred between 5-10 days after the administration of the virus. However, sensitized mice died from a smaller dose of virus. An autopsy of these mice showed that death was due to influenza infection with typical lesions of the lungs.

(2) Incubation with specific antiserum inactivated virus and rendered it non-infective for normal and for sensitized mice.

(3) Injecting antiserum intraperitoneally into normal mice produced considerable protection to intranasal inoculation of the virus.

TABLE III. Protection of Normal and Sensitized Mice by Antiviral Antibodies against Virus Administered Intranasally in Volume of .05 ml Diluted 1:20.

Serum inj. intraper. .2 ml	Mice vaccinated by *H. pertussis* vaccine	Normal (not vaccinated) mice	P value
Undil. and dil. 1:10	21/22	10/25	P <.001
Dil. 1:100	6/ 6	6/ 6	
No serum (control)	11/11	10/10	

On the contrary similar antiserum treatment gave very poor protection to the virus in sensitized mice. Apparently the state of hypersensitivity impaired the protecting qualities of the antibodies.

The observations presented in this report may have special interest for prophylactic work. Due to the present-day mass immunization of children with various bacterial antigens (including *H. pertussis*) and the possible increase of the number of cases of bacterial allergy, the regular test for the safety of a new vaccine on normal animals may not be adequate. Our studies offer a very simple technic for testing a new vaccine on hypersensitive mice.

Summary. Experiments with mice indicate that hypersensitivity induced by the *H. pertussis* antigen can increase susceptibility to influenza virus.

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New Olive Oil Emulsion for Lipase and New Observations Concerning "Serum Lipase". (22038)

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Emulsions prepared from olive oil, or similar fats, tend to separate on standing after short periods of time. For tests of lipase activity stable emulsions are required. In addition, there is in these tests a correlation between the degree of emulsification and the amount of lipase activity detected(1). In the present paper a simple method for preparing a stable emulsion of olive oil is described. With this emulsion the "lipase activity" of various sera was determined. In addition the following points were investigated: (a) the activation of serum lipase by Lima bean trypsin inhibitor, (b) a method for demonstrating and determining antilipase in serum, (c) other observations on lipase.

Methods. Preparation of a stable olive oil emulsion. The emulsion is a conventional olive oil-acacia mixture to which there is added 0.25% of a new hydrophilic colloid "Carbopol 934" (B. F. Goodrich Chemical Co.). Carbopol 934, having carboxyl groups, disperses in water to yield a low viscosity acid solution which on neutralization turns into a stable gel. Toluene (0.5%) is also incorporated into the emulsion to prevent bacterial growth. Control tests demonstrated that toluene does not inhibit lipase action. One gram Carbopol 934 and 200 ml of distilled water were mixed in Waring blendor until dispersion was complete (5 minutes). A solution of 5 g of acacia (McKesson, U.S.P.) in 100 ml of distilled water was added to the Carbopol solution; the mixture was stirred in a Waring blendor for 5 minutes and then filtered through glass wool. The blendor must be thoroughly washed at this time to remove the small particles of Carbopol which would interfere because Carbopol is a strong acid. The colloid mixture was then returned to the blendor, and 100 ml of olive oil (Fisher, U.S.P.) were added in a slow stream during 5 minutes with stirring. Without discontinuing mixing, N sodium hydroxide was then added dropwise until a pH of 7.00 was obtained. Usually 10.3 ml N sodium hydroxide were required. Finally, 2 ml of toluene were added dropwise and homogenization was continued for 15 minutes. Air from the emulsion was removed in a desiccator with the aid of a pump. The emulsion may be kept in a refrigerator at 4° for several weeks without separating and without an appreciable increase in its titration blank. Brief shaking of the emulsion, however, is advisable before it is used. Digests prepared from this emulsion did not separate during the digestion period. When Carbopol was omitted from the emulsion the olive oil separated within a few hours. Cotton seed oil, as marketed in this country, cannot be used as a lipase substrate since it contains natural lipase inhibitors. *Phosphate Buffer.* 5.54 g of monobasic potassium phosphate and 3.41 g of anhydrous dibasic sodium phosphate were dissolved in distilled water and made up to 1000 ml. A few drops of toluene were added to prevent bacterial growth. The pH of buffer was 6.56. *Enzyme Preparations.* Human plasma cholinesterase was prepared from Cohn fraction IV-6 by Cutter Laboratories. It possessed 800-times the cholinesterase activity of whole plasma. Wheat germ lipase was obtained from Worthington Biochemical Corp.; the pancreatic lipase from Nutritional Biochemical Corp. Normal human sera were obtained from nonfasting blood donors (North Carolina Memorial Hospital); the other sera from nonfasting animals. *Crude Lima bean trypsin inhibitor* (*LBI*). This globulin fraction of Lima beans was prepared according to Tauber, Kershaw and Wright(2).

The lipase test. 1-ml portions of serum or enzyme solution were placed in 30 x 125 mm Pyrex test tubes. To each tube 5 ml of olive oil emulsion and 0.5 ml of phosphate buffer were added. The tubes were stoppered and shaken for 1 minute and then placed in a constant temperature water bath for 20 hours at 37°. Controls were prepared by adding the serum or enzyme solution to the olive oil emulsion-buffer mixture at the end of the digestion period. The control values were subtracted from the total values. At the end of 20 hours 3 ml of 95% ethyl alcohol and 2 drops of a 1% solution of phenolphthalein in ethyl alcohol were added. After mixing, the digests were placed in 30-ml beakers and titrated with 0.05 N sodium hydroxide. A 2-ml Koch micro burette was used to which soda lime tubes were attached to prevent absorption of CO_2 by the alkali. The mixtures were stirred with a glass rod after the addition of each drop of alkali. The solutions were titrated to a deep pink color, and the pH's (9.70-9.80) were checked with a Beckman Model G pH meter. All values are expressed in ml 0.05 N sodium hydroxide. *Kinetics of serum lipase.* Hydrolysis of the olive oil within 48 hours was fairly linear with respect to time. The following titration values (minus controls) were obtained: 24 hours, 1.55; 48 hours, 2.90; 72 hours, 3.92. The lipase activity was not proportional to increasing quantities of rabbit serum. 0.1 ml serum gave a value of 0.74; 0.2 ml gave a value of 1:00; and 1.0 ml gave a value of 1.65.

Keeping quality of serum lipase. Serum lipase appears to be a fairly stable enzyme. Different sera, when kept for 24 hours at 37°, lost none of their lipase activity when compared to specimens kept in deep freeze at –18°, and at 4° for the same length of time.

Serum antilipase. The presence of antilipase in inactive serum may be demonstrated by adding some of the inactive serum to some of the active serum. For instance, when 0.2 ml of inactive guinea pig serum was added to 1 ml of active rabbit serum the titration value dropped from 1.41 to 0.45 ml. In sera that have lipase activity the antilipase may be demonstrated by keeping an aliquot of the serum for 30 minutes at 56°. This destroys all but a trace of the lipase but not the antilipase. In the antilipase test, for convenience and because of its fairly high lipase content, dilute rabbit serum may be employed as the lipase source. The rabbit serum is diluted about 10 times in order to give a titration value of 1.2 ml with 0.05 N sodium hydroxide, after 20 hours. 0.4 ml of heated human serum is added to the dilute rabbit serum. The serum mixtures are made to 1.6 ml with 0.85% sodium chloride. After 30 minutes of interaction, the lipase test is applied. Inhibition of dilute rabbit serum by different quantities of "inactivated serum," however, is not quite proportional to the quantity of inactivated serum employed. This is probably due to the presence of a trace of active lipase in the heated serum. *Inhibition of serum lipase by the antilipase of the same serum* may be demonstrated by adding 0.4 ml of heated rabbit serum (30 minutes at 56°) to 1 ml of 10-times diluted rabbit serum. Here the inactivated heated serum, containing the antilipase, brought about almost complete inhibition of the unheated serum lipase. The olive oil test was done in the usual manner. *Hemoglobin, a lipase inhibitor.* Hemoglobin is also a powerful inhibitor of serum lipase. Saline-washed rabbit red cells were frozen, thawed, laked with 20 volumes of distilled water and cleared by centrifuging. The lipase activity of 1 ml of rabbit serum was lowered from 1.54 to 0.73 when 0.2 ml of the laked rabbit red cell solution was added. For this reason sera obtained from hemolyzed blood cannot be employed in lipase assays.* *Lipase activity of different biological materials obtained with olive oil emulsion.* The following lipase values were obtained: human serum, 0.00-0.84 (average 0.22); rabbit serum, 0.50-1.70 (average 1.37); guinea pig serum, 0.00-0.25 (average 0.27); rat serum, none. Sixteen samples of human sera and 10 each of the other species, were employed. For other enzymes the following titration values were obtained: pancreatic lipase, 1.05; human plasma cholinesterase, 0.20; wheat germ lipase, 0.60. The digests contained 1 ml serum or 1 ml (20 mg) of enzyme solution, and 0.5 ml phosphate buffer. The digestions were carried out in a constant temperature water bath for 20 hours at 37°, except the pancreatic lipase-containing digest which was kept for 30 minutes in a constant temperature water bath. Only the water soluble portion (the filtrate) of the pancreatic lipase was used. The final pH of the serum digests was 7.00; the final pH of the other digests was 6.3.

Activation of serum lipase by crude Lima bean inhibitor (LBI). Table I shows typical experiments concerning the activation of serum lipase by the Lima bean inhibitor. This globulin fraction of Lima beans is a potent inhibitor of crystalline trypsin, a growth inhibitor of mammals(2), and an inhibitor of fibrinolysin(3). It was of interest to find out how lipase activity would be affected by LBI. It is seen in Table I that LBI is a powerful activator of human, rabbit, horse, and rat serum lipase. Boiling the LBI destroys some of its activity. LBI itself had no lipase activity. The activating action on serum lipase by the LBI cannot be explained at this time. Isolated serum proteins had no activating effect.

TABLE I. Activation of Serum Lipase by LBI.

		Titration values, ml		
Type of serum	LBI, mg	Total	Minus control	Increase
Rabbit*	0	2.50	1.40	
"	1	3.00	1.90	.50
"	4	3.52	2.42	1.02
"	5	3.50	2.40	1.00
"	10	3.50	2.40	1.00
"	5†	2.77	1.67	.27
None	20	1.16	.06	
"	0	1.10 (control)		
Human*	0	1.58	.48	
"	5	2.35	1.25	.77
Horse (fasted)	0	1.20	.10	
"	5	1.95	.85	.75
Rat (fasted)‡	0	1.12	.02	
"	5	1.70	.60	.58

* Pool of 6 different specimens.

† Boiled 10 min.

‡ Pool of 18 specimens.

The digests contained 1 ml of serum. The LBI was dissolved in saline solution. The digests without LBI contained saline solution instead.

Summary. A stable olive oil emulsion has been described. Using this new emulsion the lipase activity of a series of biological materials have been studied. Sera of different mammals showed different lipase activities. Sera of some species had a wide range of activity, whereas, those of others had no activity. Wheat germ "lipase" splits olive oil only slightly. Highly purified plasma cholinesterase was almost inert indicating that this enzyme is not involved in fat digestion. Hemoglobin is a powerful inhibitor, and Lima bean inhibitor, a powerful activator of serum lipase. A method for demonstrating antilipase in serum has been presented. It is well known that normal human serum and the

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* We are indebted to Dr. Paulo Seabra for calling this fact to our attention.

serum of most other mammals do not contain a specific lipase similar to pancreatic lipase. The hydrolysis of olein described in this paper is affected by another type of enzyme. For convenience the term lipase has been employed in this paper.

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Variation in Response of Various Mouse Strains to Hexobarbital (Evipal). (22039)

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The use of inbred strains of mice, as an established experimental procedure in areas of medical research other than cancer, has been less extensive for various reasons, particularly because of the lack of information concerning characteristics of these established strains other than tumor type and incidences. Various investigators have determined some of the other characteristics these strains exhibit. Russell, Newfeld, and Higgins(1) have investigated the normal blood picture in 18 inbred mouse strains; Law, Morrow, and Greenspan (2) have investigated the liver glucuronidase activity in inbred strains; Tuba(3) has determined serum tributyrinase levels in 3 inbred strains; and Wragg and Spiers(4) have demonstrated inbred strain differences in response to adrenal cortical hormones.

The data herein reported is additional information on strain differences in mice, and is concerned with the response to a barbiturate drug, hexobarbital (Evipal), as measured by sleeping time.

Materials and methods. The various strains tested, shown in Table I, came from the production colonies of the National Institutes of Health. Initially, the investigation was concerned only with closely inbred strains, but because of the existence of some doubt about the uniformity of inbred strains in research of this sort(5), it was decided to extend the observations to additional strains in order to test other genetic situations. The first 10 strains listed in the table have been closely inbred by brother x sister matings, so they represent a genetic condition in which maximum homozygosity is to be expected. The CFW strain has been mated brother x sister in a large closed colony with no effort made to maintain close relationships between matings, so that many family lines are maintained parallel in each animal generation. Genetically, such a population will no doubt exhibit considerable homozygosity, but is expected to be more variable than the more closely inbred strains. The non-inbred Swiss strain has been random

TABLE I. Mean Sleeping Time in Minutes for Various Strains of Mice Injected with 125 mg per Kilo Body Weight of Evipal. All animals were males, 70-80 days old.*

Strains	No. of animals	Mean sleeping time	Stand. dev.
A/LN	25	48.44	4.17
DBA/2JN	64	46.83	4.22
A/HeN	25	41.25	1.92
BALB/cAnN	63	41.01	1.99
C57L/HeN	29	32.52	3.22
C57BL/6JN	37	32.26	5.71
C57BR/cdJN	25	31.93	5.51
C3HfB/HeN	30	22.02	2.99
BRSUNT/N	26	18.34	3.34
SWR/HeN	38	18.08	4.15
CFW	32	46.43	11.75
Swiss (non-inbred)	47	43.39	14.51

* The author wishes to thank Dr. Marvin Schneiderman, National Cancer Institute, for his advice on statistical procedures.

mated within a large closed colony, and hence represents considerable heterozygosity. All animals were 70-80 days old when tested, and only males were used. The barbiturate, Evipal, was injected intraperitoneally at a dose level of 125 mg per kilo of body weight. Sleeping time was measured in minutes from the time the injection was made until the righting reflex was restored. The test for significant differences between sleeping time means was made according to the method of Baten(6).

Results. Table I shows the results of sleeping time determinations in various strains of mice. As the data show, strain differences in response to the drug are evident. The A/L and DBA/2 strains sleep the longest with no significant difference existing between the 2 sleeping times. Likewise, the A/He and BALB/c strains fall into a second group with no difference between the 2, but both differing significantly from the A/L and DBA/2 strains. The 3 C57 strains fall into a third group with no difference between the 3, but differing significantly from the first 4 strains. The C3HfB is similar in sleeping time to the BRSUNT and SWR, but differs significantly from these 2 strains. The CFW and Swiss have long mean sleeping times, comparable with the A/L and DBA/2 strains.

The uniformity of the distributions of the various strains is of particular interest. An examination of the standard deviations will readily show that the distributions of the inbred strains are considerably less variable than those of the CFW strain, and it in turn is less variable than the non-inbred Swiss. This latter strain was quite variable, ranging from one animal that was completely unaffected to 2 animals that slept 97 minutes. Thus, 3 different levels of genetic homogeneity have exhibited 3 different levels of variability, with the closely inbred strains showing minimum variation at this particular injection level. Whether this will hold true for other injection levels is not known. However, in this regard, preliminary trials with injection levels of 25 mg, 50 mg, and 75 mg per kilo of body weight showed considerable variation in response by all the strains. More complete information on this point is not yet available.

Discussion. The significance of the differences in mean sleeping time between the various strains tested is not apparent at this time. Whether this indicates differences in sensitivity to hexobarbital, or differences in the rate of biotransformation is yet to be determined. Quinn, Axelrod, and Brodie(7) have found an inverse relationship between the rate of biotransformation of hexobarbital and the duration of its hypnotic effect in a number of species. Perhaps a similar relationship exists in these various strains of mice. The fact remains, however, that strains do differ in their response to this drug, and these differences are consistent and uniform for the strain. Therefore, a genetic mechanism is probably involved.

Strain differences in response to various drugs could be of considerable importance in routine pharmacological testing of drugs, or in basic studies of drug metabolism systems. It is quite likely that one approach to the study of metabolic pathways in small mammals can be made by the use of genetically uniform animals in combination with pharmacological technics.

The demonstration of strain differences in response to such a physiological condition is interesting from a genetic standpoint, for it supports the concept of the uniformity of response of closely inbred strains, a concept that has been the backbone of the creation and use of inbred strains for many years. Furthermore, the demonstration that the uniformity of response corresponds to the theoretical state of homozygosity, supports the concept even more strongly. However, in this regard, recent work by McLaren and Michie(6) using pentobarbital sodium (Nembutal), indicated the inbred strain tested was just as variable as an F1 hybrid of that strain and as a non-inbred strain. The physiological action of these 2 chemically similar drugs has always been considered similar, so whether this discrepancy in results is due to an actual difference in action, or difference in dose levels, or differences in the animals used cannot be determined at this time. Certainly additional work on drug metabolism in the various inbred and non-inbred strains of mice is indicated.

Cooper and Brodie(8) have recently demonstrated an enzyme system localized in the microsomal fraction of liver cells, which oxidizes hexobarbital to keto-hexobarbital. The investigation of the activity of this enzyme system in these strains is now underway.

Summary. 1. Significant differences in sleeping time have been demonstrated between various strains of mice, inbred and non-inbred, when injected with 125 mg per kilo body weight of hexobarbital. 2. Three different levels of genetic homozygosity were represented in the various strains tested. The most closely inbred strains (A/L, DBA/2, A/He, BALB/c, C57L, C57BL/6, C57BR/cd, C3HfB, BRSUNT, SWR) showed the least variation, the less closely inbred strain (CFW) showed more variation, and the non-inbred Swiss strain showed the most variation.

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Blood ACTH Levels Following Subcutaneous Injections.* (22040)

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The adrenocorticotropic hormone (ACTH) is known to disappear rapidly from the circulating blood. Van Dyke *et al.*(1) have estimated the average life of the ACTH molecule in the circulation to be approximately 17 minutes, and half-life values of about 5 minutes have been estimated for ACTH administered intravenously to intact rats(2,3). The half-life of endogenous ACTH in the adrenalectomized rat has been reported to be approximately one minute(4). A rapid rate of disappearance of ACTH from the circulation is also indicated by clinical studies. Intravenous infusion of ACTH over a prolonged period brings about a much greater adrenal response than the single injection of a comparable dose of the hormone(5). It becomes of interest, therefore, to establish the rate at which exogenous ACTH disappears from the circulation following a single extravascular administration.

In these studies a large dose of ACTH was administered subcutaneously to hypophysectomized rats and blood ACTH levels were estimated at various intervals after treatment.

Method. Male hypophysectomized rats were obtained from the Hormone Assay Laboratories, Chicago, and used within a 24-hour post-operative interval. An ACTH standard dose response curve was prepared for this study based on accumulated data representing

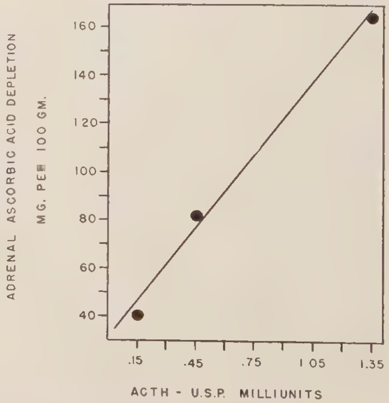

FIG. 1. ACTH standard dose-response curve. Each point represents the mean response of 25 hypophysectomized rats 1 hr after intrav. injection with U.S.P. ACTH Reference Standard.

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TABLE I. Depletion of Adrenal Ascorbic Acid in Recipient Rats following Injection of 4 ml of Blood from Donor Rats Previously Injected with 10 U.S.P. Units of ACTH.

Min. after subcut. inj. of donor rats	No. of rats	Adrenal ascorbic acid depletion (mg/100 g) of recipient rats 1 hr after intrav. inj.	Blood ACTH of donor rats (mU/4 ml)*
1	6	77	.44
2	6	90	.56
4	4	134	1.00
8	5	119	.88
10	3	106	.76
16	6	123	.92
20	3	43	.12
32	3	69	.36
40	3	58	.32
80	3	10	.00

* Obtained by interpolation of column 3 using Fig. 1.

25 rats at each point injected intravenously with U.S.P. ACTH Reference Standard (Fig. 1). Measurements of blood ACTH were made by the technic of Sayers and Burke(6). Hypophysectomized rats, 250 g, were injected subcutaneously with 10 U.S.P. ACTH units in 0.5 ml water. At various time intervals following treatment 4 ml of blood from a previously injected rat were collected from the abdominal aorta into a heparinized syringe. The blood was immediately injected into the jugular vein of a recipient 100 g hypophysectomized rat. The donor rat was then discarded. Individual rats were used for each blood collection. Adrenal ascorbic acid of recipient animals was determined 1 hour after the transfusion. These values were subtracted from uninjected control values to estimate adrenal ascorbic acid depletion. Blood ACTH values were obtained by interpolating the depletion values on Fig. 1.

Results. Table I presents the ascorbic acid depletion values found in recipient rats receiving 4 ml of blood from donor animals.

The concentration of ACTH in each ml of rat blood (obtained from Table I) is shown graphically in Fig. 2. Detectable ACTH activity is found in the blood within one minute after subcutaneous injection.

The blood ACTH level rises rapidly and reaches a peak concentration between 4 and 8 minutes after injection. After 20 to 40 minutes the blood ACTH is almost back to zero and 80 minutes after injection no detectable activity is found.

In a separate experiment, adrenals were removed from the donor rats at the time that blood sampling took place. The adrenal ascorbic acid depletion was determined and is shown plotted with blood ACTH levels in Fig. 3. Significant depletion of adrenal ascorbic acid in the donor rats does not occur until about 28 minutes after the peak level of ACTH in the blood is reached.

Discussion. The present data indicate that exogenous ACTH, even when administered subcutaneously in a large dose rapidly disappears from the blood. Endogenous ACTH, too, disappears quickly from the blood after ether anesthesia(6) and after scald(7).

Pincus *et al.*(8) showed that up to 70% of added ACTH activity is lost within 1 hour from blood. Since such *in vitro* inactivation may be prevented by previously heating the blood(8), it is likely that at least some of the disappearance of ACTH from blood *in vivo* may be due to enzymatic inactivation.

The time relationship between ACTH administration and adrenal ascorbic acid depletion has been the subject of many studies (9,10), but this is believed the first investigation where the actual ACTH concentration in the blood was correlated with its corresponding adrenal ascorbic acid depletion. This is significant since it shows that despite an unphysiologically high ACTH blood concentration a 28-minute lag in the adrenal response still occurs.

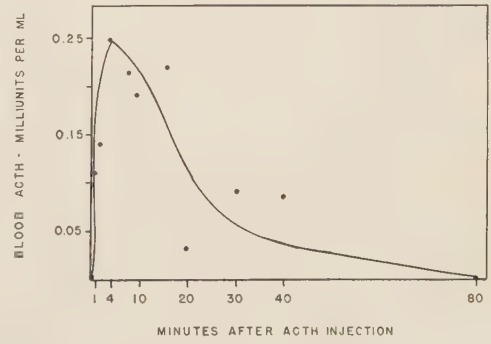

FIG. 2. Blood levels of ACTH in hypophysectomized rats after a single subcut. injection of 10 U.S.P. ACTH units. Each point represents mean response of 3 to 6 animals.

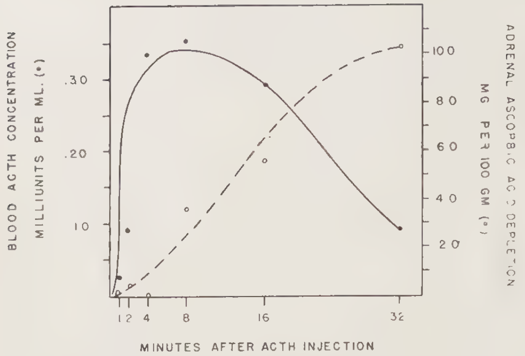

FIG. 3. Simultaneous measurement of blood ACTH concentration and adrenal ascorbic acid in hypophysectomized rats treated subcut. with 10 U.S.P. ACTH units. Each point represents mean response of 3 animals.

The highest blood concentration of ACTH observed in this study (Fig. 3) was 0.35 milliunit per ml blood. The blood volume of a rat is estimated to be 6 ml per 100 g(11,12). In the 250 g rats used in these experiments a total of only 6.1 milliunits, therefore, appears to be present in the circulation at the time of the highest blood concentration out of 10,000 milliunits injected. This indicates massive inactivation of the hormone at probably both the site of injection and after it enters the circulation.

Summary. The rate of disappearance of exogenous ACTH from the blood in the hypophysectomized rat has been determined. Following subcutaneous injection of 10 U.S.P. ACTH units detectable blood ACTH activity was found within 1 minute. A peak concentration of 0.25 to 0.35 milliunit per ml blood took place 4 to 8 minutes after injection. No detectable activity was found after 80 minutes. Significant adrenal ascorbic acid depletion did not occur until about 28 minutes after the peak concentration of ACTH in the blood was reached.

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Pathogenesis of Fatal Encephalomyocarditis (EMC) Virus Infections in Albino Rats. (22041)

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This paper presents a study of encephalomyocarditis virus infections, carried out by means of virus titrations and histopathologic studies of various organs of albino rats moribund from disease. EMC virus can produce paralysis and fatal encephalitis in albino rats although other species of rats and mongooses, when inoculated with this agent, appear to die principally from myocarditis(1). The subject has additional interest in that previous investigators(2-6) have reported only inapparent infections in albino rats.

Materials and methods. Experimental animals. Albino rats were random bred. Some were originally obtained from Makerere College Medical School and others from Veterinary Research Laboratory, Kabate, Kenya. Hooded rats, which originated at Lister Institute, Elstree, England, were employed for a few experiments. Mice were of the Swiss albino strain. *Virus.* Of 3 strains of EMC virus employed, 2 had been isolated from animals in monkey runs of the Virus Research Institute. The principal one we used was originally obtained from an infected baboon and had been carried through 6 intracerebral passages in mice; the second strain was isolated from a rhesus monkey and had been passaged 16 times in mice; the third strain was one originally ioslated from a chimpanzee in Dania, Florida(7), and had been passed 78 times in mice. This last strain was obtained from Dr. Karl Habel, National Institutes of Health, Bethesda, Md. The rhesus and Florida strains were studied for comparative purposes. *Virus isolations from inoculated animals.* Ten percent tissue suspensions in meat infusion broth containing penicillin and streptomycin were centrifuged at 4000 rpm for ½ hour prior to intracerebral inoculation of 0.03 ml into mice 5 weeks of age. For titrations, groups of 4 mice were used for each 10-fold dilution. Blood obtained by cardiac puncture was heparinized to prevent clotting. Urine, aspirated from the bladder at necropsy, was obtained from only a few animals. Samples of gut were removed routinely from the lower ileum and laid open and flushed free of intestinal contents in 3 changes of broth. Formed feces were obtained by incision of the rectum. Storage for all specimens was at –20°C. Under these conditions, little or no diminution of virus titer in frozen specimens appeared to take place within 4 months. A majority of suspensions, nonetheless, were titrated in mice shortly after being harvested. Inoculated mice were then observed for 8 days for paralysis and death. Specificity of illness induced was judged by a characteristic incubation period of 3-5 days and the clinical picture, of which paralysis of the hind legs was a cardinal feature. Fifty percent end-points were calculated by the method of Reed and Muench(8). *Neutralization tests.* Spot identifications of viruses isolated were made by means of neutralization tests performed with normal and EMC-immune rabbit sera. The antiserum was prepared by immunization of rabbits with brain suspensions of mice injected with the Florida strain of EMC virus. All sera were inactivated at 56°C for ½ hour prior to mixing with an equal volume of virus suspension that would give final dilutions containing 100 or more LD_{50} of virus. Serum-virus mixtures were inoculated into mice intraperitoneally in volumes of 0.1 ml after ½ hour of incubation at room temperature.

Results. EMC virus (baboon strain) was carried through 15 passages in albino rats 3 to 5½ weeks of age by intraperitoneal inoculation of 10% suspensions of brains of ill rats. Repeated passage did not result in any ob-

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* Work done while on excused absence from the U. S. Department of Health, Education, and Welfare, Public Health Service, National Microbiological Institute, National Institutes of Health, Bethesda, Md. Present address: Rocky Mountain Laboratory, Hamilton, Mont.

TABLE I. Virus Titrations of Various Organs in Albino Rats Infected with EMC Virus. Rats were sick 24 hours or less when sacrificed.

Rat	Strain of virus	Age inoc. in days	Blood	Brain	Fem. mus.	Heart	Spleen	Ileum	Feces	Urine	Incubation period, days
113W	Baboon	5	4.6*	7.7	4.5	2.7	2.3	—	—	—	2
114W	”	11	3.7	7.5	5.3	3.5	3.3	4.5	0	0	3
47W	”	25	<1	4	1	1	1	0	0	—	3
117W	”	32	<1	5.2	0	1	1.5	0	0	—	4
133W	”	72	0†	4	0	<1	<1	0	—	—	9
127W	Florida	30	0	4.5	1.4	0	1.5	0	0	—	4
128W	Rhesus	29	0	5.5	0	2.5	0	0	0	—	5

* Titers expressed as inverse log of dilution killing 50% of mice inoculated.

† 0 = No virus isolated from 1:10 suspension.

served change in virulence. Although rats of all ages might become paralyzed and die, susceptibility, which was 100% in suckling animals, became increasingly unpredictable in rats over 3-4 weeks of age. Severe crippling of one and frequently of all 4 extremities gave rats a helpless appearance and occasional rats had a hemorrhagic, watery exudate along the eyelids. Death usually supervened in one to 3 days. The incubation period was 2-3 days in rats under 2 weeks of age, and 4-6 days, rarely up to 11 days, in older rats. Table I presents comparative titrations of various tissues from albino rats of increasing ages which were killed when moribund. All, except for 47W, were given the same inoculum of 12th passage rat brain intraperitoneally. Suckling rats (113W and 114W) had considerable amounts of virus in all organs tested with highest titers in brain tissue, whereas the older rats (47W, 117W, and 133W) had little recoverable virus from any organs except brain. Seventeen other rats, from 22 to 39 days of age and representing 4th to 14th passages of EMC virus, were also sacrificed when moribund and studied by methods similar to those just described. Although titrations in many cases were limited to blood, brain, and femoral muscle, titers obtained were essentially the same as those obtained for rats 47W, 117W, and 133W (Table I). None of these older rats exhibited the widespread distribution of virus encountered in suckling animals. Two rats, 3 to 4 weeks of age, inoculated intraperitoneally with 13th-passage brain suspensions, developed paralysis of the hind legs within 4 days. Brains, when harvested and pooled, had a titer of $10^{-4.5}$. Lumbar spinal cord from one rat had a titer of $10^{-2.5}$ and from the other of $10^{-2.8}$.

Principal histologic changes encountered in limited examinations of sick rats were observed in the brain. In brains of 3 rats examined, lesions consisted of scattered foci of degeneration and cellular infiltration with lymphocytes and macrophages. No study was made of the localization of these lesions. Lesions were also found in spinal cords from 3 rats examined. These included focal hemorrhages, perivascular infiltration with polymorphonuclear leucocytes, lymphocytes, macrophages, and degeneration and loss of nerve cells. Definite myositis was found in both of two rats examined. These rats, inoculated intraperitoneally at 3-4 weeks of age, had developed crippling of the extremities 5 days later. As illustrated in one rat (Fig. 1), lesions included degeneration of muscle fiber, disappearance of fiber, and a mild infiltration of round cells and polymorphonuclear leucocytes.

Viremia. Albino rats, other than suckling animals, exhibited minimal or no viremia. To determine whether viremia of greater degree might take place during the incubation period, 9 rats 26 days of age, were inoculated intraperitoneally. At 7 hours after inoculation and then daily for 6 days, blood was obtained from alternating pairs and pooled for testing. No virus was recovered from 1:10 suspensions of blood obtained from well rats during these periods, although 7 of the 9 eventually became paralyzed. Blood obtained from a pair with paralysis 3 days after inoculation had a virus titer of $10^{-2.7}$. Two other pairs of rats, however, which became ill 5 and 6 days after in-

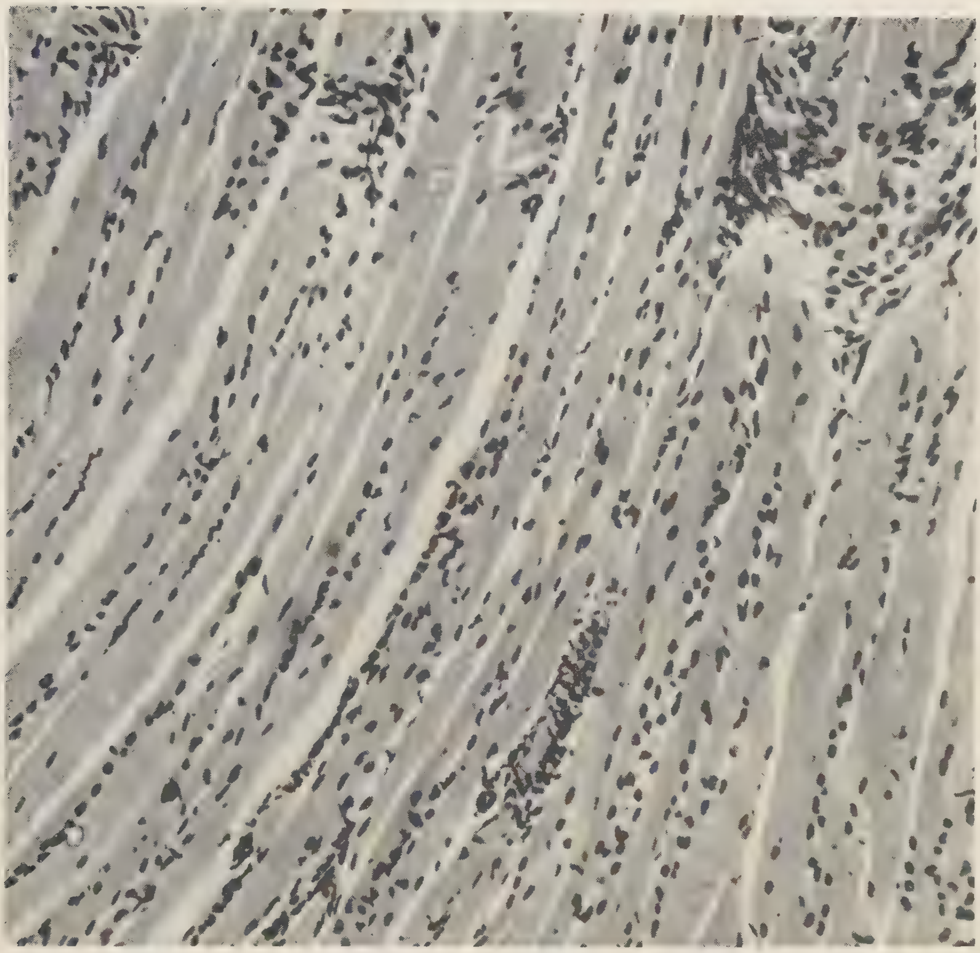

FIG. 1. Myositis in rat skeletal muscle (H & E × 170).

oculation, had no demonstrable viremia.

Inoculation of pregnant rats. Experiments on pregnant rats were performed to determine 1) if disease might be transmitted to offspring and 2) whether this virus, by growth in uterine muscle, might interrupt pregnancy. Seven rats, presumed to be pregnant, were inoculated intraperitoneally. Four of these gave birth to healthy litters 4 to 6 days after inoculation. Mothers and offspring remained well. Two rats, which proved not to be pregnant, developed paralysis of the hind legs, one in 7 days (weight 132 g) and one in 11 days (weight 236 g). Brains of both contained virus in high titer. The 7th rat gave birth in 3 days and at 7 days after inoculation she began dragging her hind-quarters but was still nursing. Of mice inoculated with a 1:10 brain suspension from this rat, only one of 3 died. Such a minimal amount of virus in an inoculated animal was regarded as being of questionable significance. No virus was recovered from a young one sacrificed a day after the mother became ill.

Ingestion and inhalation of virus. Albino and hooded rats were susceptible to infection both by ingestion and by inhalation. They are considered together, since no strain difference in susceptibility was ever apparent throughout present experiments. Of 18 rats 25 to 29 days of age inoculated intranasally with infected rat brain suspensions containing 10^{-3} to 10^{-6} LD_{50} of virus, 9 became paralyzed and died within 4 to 11 days. Two of the ill rats were tested for virus which, though readily recovered from other organs, was not found in lung suspensions. Rats became ill less readily as a result of ingestion. Thus, 23 rats 3 to 4 weeks of age were fed infected carcasses of rats and mice. Of these, 2 were subsequently found dead and 2 developed paralysis, one in 6 days and another 2 weeks after feeding. EMC virus was recovered from brains of the ill rats. Seven of the survivors, tested for neutralizing antibodies 2½ to 3 weeks after an infective meal, were negative.

Comparative effect of other strains of EMC virus. A final question arose as to whether findings obtained in albino rats resulted from use of a particular strain of EMC virus, the one originally isolated from a sick baboon. For comparative purposes, the 2 other strains were inoculated into albino rats. These strains killed all rats under 2 weeks of age. Of 4 rats, 29-30 days of age, inoculation of the Florida strain intraperitoneally led to death of 2 and paralysis of another within 4 days. Inoculation of 3 rats of similar age with the rhesus strain led to paralysis of all in 5 days. Two rats were alive but crippled after 14 days. One acutely ill rat from both series was sacrificed. Patterns of virus distribution (rats 127W and 128W, Table I) did not differ essentially from those observed in albino rats inoculated with the baboon strain. Myocardium of rat 128W, however, had a titer of $10^{-2.5}$. Possibly this relatively high titer was related to the fact that the rhesus strain of EMC virus, as used in this experiment, had been previously passed through 7 intraperitoneal passages in multimammate rats(1).

Discussion. Induction of paralysis and death in albino rats is in contrast to reports of others(2-6) who described only inapparent infections. Although it is difficult, if not impossible, to explain differences in results, several factors should be considered. One is that only suckling rats are fully susceptible. A lesser number of older rats develop frank disease. As a rough estimate, based on overall experiments, 30% of rats inoculated intraperitoneally at 30 days of age and 10% of rats inoculated when 60 days or older might be expected to develop paralysis. These percentages would doubtless be increased by intracerebral inoculations. A second factor might be that some stocks of albino rats are resistant. Our results, however, were obtained with 3 different strains obtained in Africa and, in an additional experiment, non-inbred albino rats (Osborne-Mendel strain) from the National Institutes of Health, Bethesda, Md., proved susceptible to EMC virus. Strain of virus did not appear to be a factor of importance, since EMC virus originating from Dania, Florida, also led to death and paralysis. The widespread infection with viremia encountered in suckling rats and the limited distribution of virus in adults were findings somewhat parallel to those described by McLaren and Sanders (9). These investigators found that EMC virus grew well in muscle and brain of 5-day-old mice, but not as well in muscle af 30-day-old mice, although brains of the latter remained fully susceptible.

A question of importance is to what extent are titers the result (a) of intrinsic growth of virus within an organ and (b) of residual blood contained in the organ. If titers were secondary effects of a viremia, one would expect to observe 2 types of relationship. First, blood-containing organs such as the spleen should have relatively high titers and, second, any excised specimen of muscle or washed piece of ileum might be expected to have a titer at least 2 logs lower than that of the blood, if one may make estimates from blood volumes calculated by Kaliss and Pressman (10) for mouse organs in which blood vessels were tied off. As shown in Table I for suckling rats, virus presumably multiplied in all organs tested.

Summary. 1. EMC virus can produce a crippling and usually fatal illness in albino rats of all ages. 2. Suckling rats are more susceptible than older rats and experience a greater degree of systemic infection. 3. In older rats, the disease is chiefly localized in the central nervous system, although myositis was likewise observed.

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Glucagon Content of Pancreatic Tissue Devoid of Alpha Cells.* (22042)

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(Introduced by G. L. Duff.)
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In spite of the large amount of work done in the past 30 years to determine the site of origin of glucagon, there is as yet no general agreement as to whether or not glucagon is the alpha cell hormone(1-5). Some have considered that the alpha cell is the source of glucagon because of differences in insulin requirement of alloxan(6) and metahypophyseal diabetes(7) as compared to that of surgical diabetes(8). This opinion is supported by the presence of a hyperglycemic factor in extracts from normal, alloxanized, and alloxanized duct ligated pancreas(3). An alternative explanation for the differences in insulin requirement was that in surgical diabetes intestinal absorption of nutriments is limited(9). This latter view was controverted by other experiments with duct ligated alloxan diabetic dogs(10). A hyperglycemic factor, apparently identical with glucagon, is present in the stomach of the dog. This was considered to confirm the origin of this substance from the alpha cell of the pancreas because of supposed similarity of argentaffin cell of the stomach with the alpha cell(1). Furthermore in one report, gastro-intestinal argentaffin cells are implicated in disturbances in carbohydrate metabolism(11). However, other workers have not found any correlation between tumours of these cells and diabetes(12). Similarly, glucagon could not be extracted from the stomach of the hog despite the presence of argentaffin cells(1). The lack of identity between the alpha cell and the intestinal argentaffin cell, originally demonstrated by Masson in 1924, has recently been confirmed(13, 14). It would therefore be erroneous to consider that the presence of a hyperglycemic factor in tissues containing argentaffin cells supports the conclusion that the alpha cells serve as the source of glucagon. The discovery of chemical agents apparently capable of damaging alpha cells, rather than clarifying this problem, gave further contradictory results(3-5). Some workers reported alleviation of diabetes by cobalt(15,16), and diethyldithio carbamate(16). Others reported no effect of either of these agents or of synthalin A on the severity of alloxan diabetes (13,17,18). In some studies extracts from the pancreas of cobalt treated animals have been reported to contain lesser quantities of glucagon than normal(4,19). On the other hand, one of us(5,20,21), using qualitative methods of glucagon assay could detect no significant differences between normal and cobalt treated pancreas. Others have reported that cobalt treatment increases the glucagon content of the rabbit pancreas, while synthalin A causes its complete disappearance(22). In this connection, it is of interest that the lesions reported by various investigators after cobalt, are not always identical; it seems to vary with the animal species, the dose and the route of

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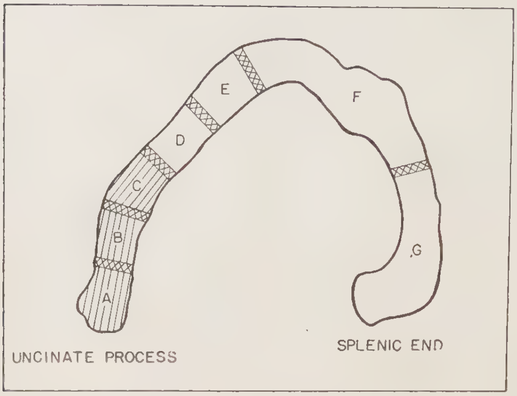

FIG. 1. Tracing of outline of dog pancreas. Longitudinally striated portions A, B and C were free of alpha cells while the remainder contained these cells. Cross hatched areas indicate tissue examined histologically.

administration(4,5,13,23,24).

As was previously pointed out, it has not been possible by the use of cobalt to prepare pancreas which are completely devoid of alpha cells(4,5). However, it has recently been demonstrated in this laboratory that the dog pancreas can be divided into two portions; one which does not contain alpha cells and which corresponds roughly to the uncinate process, and the balance of the pancreas which contains alpha cells(25). This finding enables a reinvestigation of the problem of the relationship of glucagon to alpha cells, since pancreatic tissue completely devoid of these cells is available for extraction.

Material and methods. The entire pancreas was removed from 6 normal mongrel dogs weighing between 15 and 20 kg which had been killed by exsanguination from the aorta after preliminary nembutal anesthesia. A tracing of the outline of the pancreas was made and the uncinate process was then divided into portions of approximately 1 to 2.5 g each. A complete transverse section of the proximal end of each portion was taken for histological examination and the position of these biopsies was recorded on an outline of the pancreas as indicated by the example in Fig. 1. In addition, transverse sections for morphologic study were obtained from the body and splenic end of the pancreas. The portions of the uncinate process and also the remainder of the pancreas were each immediately placed, separately, in acid alcohol and minced. Extracts prepared by the method of Best *et al.*(26), were dried and kept in the refrigerator until ready for use. The tissue for histological study was fixed in Zenker-Formol solution and sections were subsequently stained with Masson's trichrome, Gomori's chrome alum hematoxylin(27), Gomori's Aldehyde Fuchsin(28) and Mallory-Heidenhain-azan(29). In addition portions of the pancreas from the uncinate process and body

TABLE I. Blood Sugar Response of Fasting Anaesthetized Normal Cats to Intravenous Injection of Dog Pancreatic Tissue. (A) Containing alpha cells; (B) Devoid of alpha cells.

Material injected	Dose, g*	Dog	Blood sugar, mg %					
			Before inj.	Min. after admin. of extract				
				5′	10′	15′	20′	25′
A. Extract of alpha cell containing portion of pancreas	1	L 9	122	172	204	200		164
	”	”	94	131	142	147	133	109
	”	”	111	180	200	202	200	145
	”	L 12	115	169	202	197	187	177
	”	L 13	137	194	200	198	192	140
	”	”	124	137	200	200	182	182
	1/10	L 12	87	117	112	82	69	65
	”	”	102	126	124	110	107	92
	”	”	114	120	136	120	107	100
B. Extract of pancreatic tissue devoid of alpha cells (uncinate process)	1	L 9	112	110	108	100	85	67
	”	”	110	115	106		92	82
	”	”	104	100	92	83	74	66
	”	L 12	112	112	108	97	88	88
	”	L 13	129	127	120	112	101	103
	4	L 12	90	103	102	76	65	53
	”	L 15, 16, 17	114	120	110	94	90	82

* Calculated in term of wt of fresh pancreas.

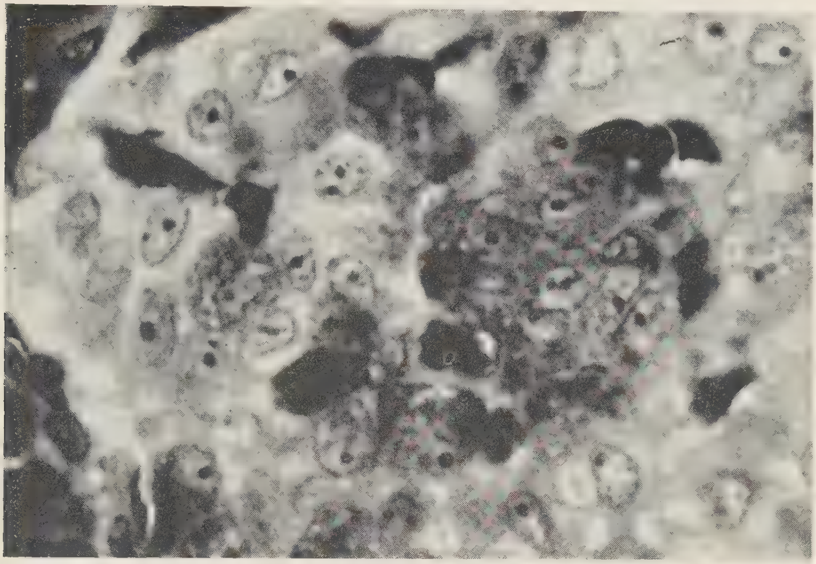

FIG. 2. Pancreatic islet from the alpha cell containing portion of pancreas. Dark finely granulated cells in center of islet are alpha cells and stained black in the original preparation. Zenker-Formol, Masson's Trichrome × 1045.

of 3 of the dogs were placed in 10% formol and impregnated with silver according to Fontana's method. For estimating the glucagon content the dried extracts were dissolved in physiologic saline (0.9% sod. chloride) acidified to pH 2.4 with HCl. One cc of solvent was used for each g of pancreas. The procedure suggested by Staub and Behrens(30) was followed. After an overnight fast, normal cats weighing between 2½ and 3½ kilo received 75 mg/kilo of sodium amytal. The inguinal veins were exposed bilaterally and duplicate blood samples were obtained just prior to, and at 5, 10, 15, 20, and 25 minutes after the injection of the test material into the contralateral vein. The blood glucose was determined by a micromodification of the Folin-Wu method(31). Extracts of 1 g of pancreatic tissue from each of the various portions were tested as per Table I. In addition 1/10 g portions of the body of the pancreas (Dog L 12) were tested in triplicate. Also, extract from 4 g of the uncinate process of Dog L 12 and a 4 g portion prepared by pooling the extracts from the uncinate process of dogs L 15, 16, 17, were used.

Results. Examination of the histological sections showed that most of the uncinate process, which amounts to approximately 15 to 20% of the total weight of the pancreas, is devoid of alpha cells. The islets of this portion are smaller than those of the rest of the pancreas and are roughly estimated to be, at most, only 1/10 of the volume of the latter. In addition, the islets of the uncinate process contain certain cells which stain red with the Masson trichrome in contrast to the alpha cells which stain black when a well ripened haematoxylin is used (Fig. 2 and 3). Argentaffin cells were demonstrated by the Fontana silver method in both portions of the pancreas, and seemed to be related to the medium sized ducts. No difference was observed in their number in either area.

The changes in blood sugar levels of the fasting anesthetized cats after the injection of each extract are represented in Table I. As was to be expected, the alpha cell containing portion of pancreas uniformly elicited an initial marked hyperglycemic response. The peak value, with 1 cc of extract, was reached at 10 to 15 minutes after injection and ranged from 53 to 91 mg% above the initial level. The blood sugar did not return to the fasting value by 25 minutes. On the other hand, one cc of extract from the portion of pancreas devoid of alpha cells did not cause an initial rise in the blood glucose concentration, except in

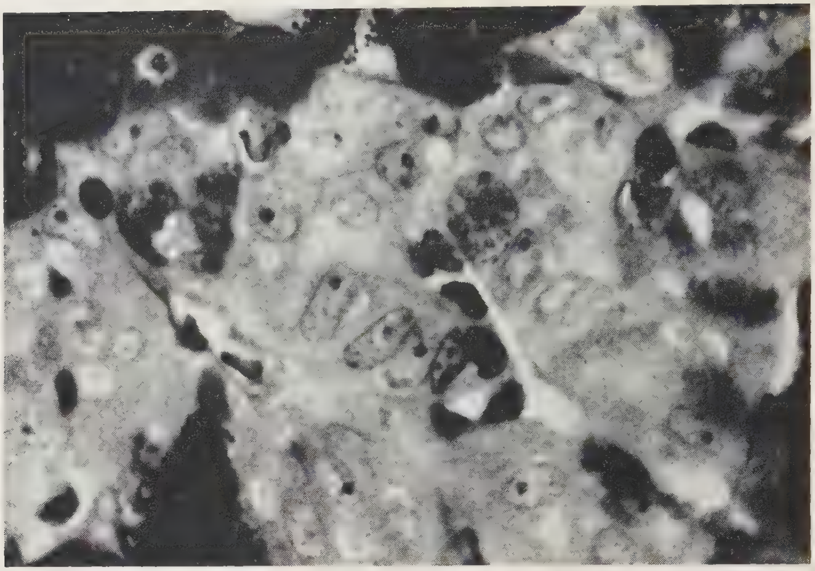

FIG. 3. Pancreatic islet from the region of pancreas devoid of alpha cells. Dark cells with rather coarse granules are X cells and stained deep red in the original preparation. Zenker-Formol, Masson's Trichrome × 1045.

one instance (L 9), where it was only 5 mg%. The 10-minute value was only 4 to 12 mg below the fasting level. By 15 minutes the insulin effect was somewhat more obvious with values of 12 mg% to 25 mg% and at 25 minutes the values varied from 24 to 45 mg% below the fasting level.

In view of the differences in islet volume observed morphologically 1/10 g equivalents of extracts from alpha cell containing portions and 4 g equivalents from the uncinate process which is devoid of alpha cells were also tested.

With the 1/10 g portions from the body, the initial hyperglycemia was less, ranging from 22 to 30 mg% above the fasting level and the peak was reached at 5 to 10 minutes after the injection. Thereafter, the blood sugar declined so that at 25 minutes the value ranged from 10 to 22 mg% below the initial level (Table I). The 4 g portion, from the areas devoid of alpha cells, also elicited a very mild initial blood sugar rise of 6 and 13 mg% above the fasting value at the 5 minutes specimen. This was followed by a decline of the blood sugar of 37 mg% and 32 mg% respectively at the 25 minutes specimens.

Discussion. The results of these experiments demonstrate an almost complete lack of hyperglycemic effect of extracts from the portion of pancreas devoid of alpha cells as compared to the remainder of the pancreas. However, the fact that a significant decline of the blood sugar did not occur until the 15 minutes specimen when 1 g portions of uncinate process were used, suggests the presence in the sample of an anti-insulin factor. The very mild initial hyperglycemia obtained with 4 g portions leads to a similar inference. Whether despite the lack of alpha cells this anti-insulin effect is due to glucagon, is open to question. Since the whole area of pancreas used for the extract cannot be examined in each case, it might be argued that some alpha cells were present. However, in other work it was demonstrated by consecutive longitudinal sections that the transition between the area devoid of alpha cells and the alpha cell containing area is quite abrupt(25). Therefore complete cross sections of the pancreas as performed in this study enable the separation of these two areas. It may be that the moderate hyperglycemia in this instance is a non-specific effect due to the large amount of foreign protein present in crude extracts.

Because of the interest in the argentaffin cells as a possible source of glucagon, these were also studied. However, only very occasional Fontana silver positive cells which identify the argentaffin cells, were found randomly distributed throughout the entire pancreas. It is therefore most unlikely that these cells do serve as a source of glucagon.

In view of the blood sugar changes obtained with the extracts from the uncinate process, it is not possible to conclude definitively that glucagon is derived from alpha cells. However, the clear differences in the results when 1 g aliquots from 2 portions of the pancreas were used, does provide evidence for the view that alpha cells produce glucagon. At the present time, no other explanation is available for these differences.

Similar conclusions have been drawn from quantitative assays of extract of cobalt treated guinea pig pancreas which showed a 60% reduction in glucagon content(31). In another study using a qualitative method of assay, a mean diminution of 10% in the hyperglycemic activity of extract of cobalt treated dog pancreas, as compared with the normal was reported(19). On the other hand no significant difference in the glucagon content of extract of cobalt treated and normal dogs were found by other workers(5,20,21). These workers stated that since many alpha cells were still left in the pancreas after cobalt treatment, and since the method of assay was qualitative rather than quantitative, a moderate reduction in glucagon content may not have become apparent. In the present study although there was a marked reduction of glucagon content in extracts from the uncinate process, the blood sugar curves from this area still indicate a minimal anti-insulin effect. This may have been a non-specific action because of the crudity of the extracts. Further assays of purified material seem to be necessary to resolve the problem of whether small amounts of glucagon are actually present in the area devoid of alpha cells.

Summary. Most of the uncinate process of the dog pancreas is devoid of alpha cells, whereas these are found in the remainder. On the other hand argentaffin cells are sparsely but evenly distributed throughout the pancreas. Extracts of the portion of pancreas devoid of alpha cells show no hyperglycemic activity when 1 g aliquots are used. When 4 g portions are employed, a very mild initial hyperglycemia does occur. These findings are interpreted to support the opinion that glucagon originates from the alpha cells but are not definitive.

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Phagocytosis by HeLa Cells and Their Susceptibility to Infection by Human Tubercle Bacilli.* (22043)

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In order for an infectious agent to multiply within its host cell it must, of course, enter the cell by passing through the cell boundary. Since this portion of the cell is relatively impermeable even to molecules of moderate size, the passage of a particle as large as a bacterium or virus might be expected to be an unusual process. In the case of some of the bacteriophages it is apparently necessary only for the nucleic acid portion of the viral particle to enter the cell, the structural material of head and tail remaining on the outside of the cell wall(1). Such dissociation of viral nucleic acid from protein may later be found also in the animal viruses, yet it seems certain that with some of the larger infectious agents which multiply intracellularly, such as the rickettsiae and the leprosy bacillus, that the infectious agent enters the cell in intact form. The mechanism by which the infectious agent passes the cell membrane would thus appear to be an important factor in determining whether or not a cell is susceptible, that is, whether the agent can multiply within the cell. One such mechanism by which the infectious agent could pass the cell membrane could be the engulfment of the particle by the host cell, and it is possible that many of the differences between the growth of bacterial and animal viruses depend upon this activity by animal cells. The engulfment of droplets (pinocytosis) has been observed in "phagocytic" cells and cells of malignant origin, including the HeLa cell, by Gey *et al.*(2) and the engulfment of particles (phagocytosis) has also been described for many animal cells. The categoric separation of cells into "phagocytes" and "non-phagocytes" is unfortunate in this connection, because the difference between cells such as macrophages and fibroblasts is in degree of engulfing activity, rather than presence or absence of this activity.

Experiments described below deal with HeLa cells and the relationship between their phagocytic activity and the culture medium placed on them. It is seen that when HeLa cells are made more phagocytic by the use of media containing certain horse serums, human tubercle bacilli quickly enter the cell where they then grow luxuriantly, even when the medium is subsequently changed to one containing human serum, in which phagocytosis is at a minimum. If one examines a HeLa cell culture a few days after it was infected with the tubercle bacilli, large increases in the amount of intracellular growth are observed to result from the use of suitable horse serum media during the first day of infection, so that in effect the HeLa cells have been rendered more susceptible to this infection.

Materials and methods. The HeLa cells were grown on cover slips in flat-sided tubes. The cultures of the HeLa cells were started from cultures in 400-ml square bottles, the growth being washed 3 times with BSS (Hanks' balanced salt solution without sodium bicarbonate) then pushed off with a rubber policeman into 5 ml of the growth medium of

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* The author wishes to acknowledge the capable technical assistance of Mrs. Mary Eleanor Jones.

Syverton, Scherer, and Elwood(3). Cell clumps were broken up with bulb and pipette and the suspension transferred to a flask containing the remainder of the growth medium, 50-70 ml per bottle. The cells in growth medium were then distributed in 1.0-ml amounts into the test tubes. After 2 days the cover slip was washed twice in the tube with 1.0 ml of BSS, 1.0 ml of the experimental medium was introduced, and 0.05 ml of the bacterial suspension added. The bacterial suspension was prepared from 4-7 day cultures of the H37Rv strain of the human tubercle bacillus grown in Dubos media containing 0.05% Tween, from which the bacilli were sedimented once in the centrifuge and resuspended in BSS by several forceful expulsions against the bottom of the tube with bulb and pipette. Phase microscopy showed the organisms to be present as individuals or small groups, and plate counts on 25% blood agar showed there were about 4 x 10^5 organisms (or clumps) in the 0.05 ml of inoculum introduced into the tissue cultures. At appropriate intervals after inoculation, coverslip cultures were prepared for microscopic study by washing twice in BSS, fixation in 10% neutral formalin, staining with carbol-fuchsin, destaining with 1% HCl in 95% ethanol, and counterstaining for 10 minutes with Giemsa. The media were changed in the remaining cultures after 2 days. All incubations were at 37°C. Suspensions of carbon were prepared from India ink according to Hanks(4). The experimental media used were variations of medium A, that is, 50% BSS and 40% serum, usually human or horse. The sera were all inactivated at 56°C for 30 minutes. Ten per cent chick embryo extract was also present in the media used in the present work, but the described effects on phagocytosis will occur in its absence.

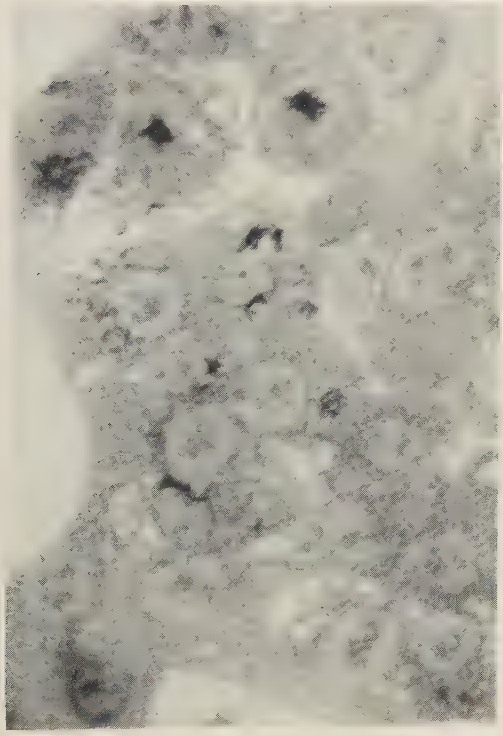

FIG. 1. HeLa cells, horse serum medium, one day after addition of tubercle bacilli. Many of the cells have taken bacilli into the cytoplasm.

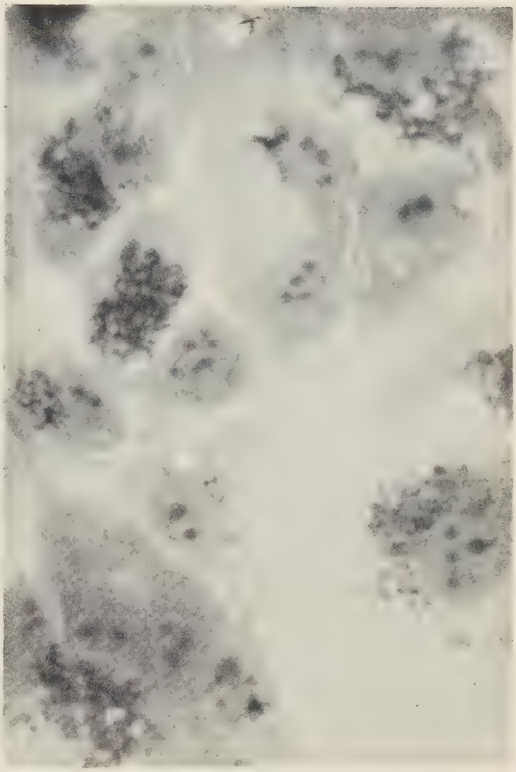

FIG. 2. HeLa cells, horse serum medium, 3 days after addition of tubercle bacilli. The bacilli in the cells have multiplied to fill much of the cytoplasm.

Results. Fig. 3 and 4 show the results obtained when the media contained human serum. Only a few cells (less than 1%) be-

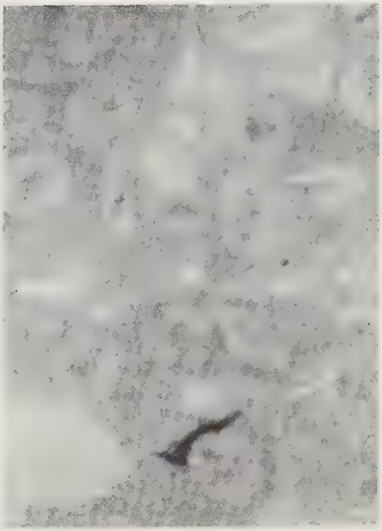

FIG. 3. HeLa cells, human serum medium, one day after addition of tubercle bacilli. Only an occasional cell has taken in bacilli.

came infected. However, when the media contained a satisfactory horse serum, 50% or more of the cells became infected (Fig. 1). Tubercle bacilli within the cells then grew rapidly so that within 3-5 days large masses of bacilli were seen in the cytoplasm(Fig. 2). Smears of the culture fluid, which were done routinely, showed that although growth occurred in the fluid with the stated inoculum, no significant difference could be noted between the horse and human serum media.

The intracellular location of the organisms as seen in Fig. 1 and 2 was evidenced by the following observations. The bacilli, after a few days, were located around the nucleus with special concentration at the cytocentrum, as is characteristic of cytoplasmic inclusions. Washing the cells with BSS and changing to fresh media of the same constitution each day did not decrease the amount of intracellular growth. The inclusion of streptomycin in the culture media in concentrations of 4 γ/ml did not affect intracellular growth, although this concentration was enough to prevent extracellular growth. A change of the medium to one containing human serum did not alter bacillary growth within the cells. Lastly, sections of infected cells cut perpendicularly to the plane of growth showed the bacilli to be surrounded by cytoplasm.

That this effect was due to increased phagocytosis rather than to an increased invasive property of the bacilli as a result of growth in the tissue culture medium is indicated by experiments with nonliving particles. If the inoculum was a suspension of heat-killed tubercle bacilli or carbon particles, or both, these particles were found within the HeLa cells in much greater numbers with horse serum media than with human serum media (Fig. 5 and 6).

A number of other culture media have been tried but none has given as much phagocytic activity as the horse serum media described. Sera from other species, monkeys, rabbits, cattle, guinea pigs, and chickens, as well as bovine amniotic fluid, were also tried as substitutions for the serum in medium A. Although some samples of chicken serum and bovine amniotic fluid showed better results than human serum, none was as good as favorable samples of horse serum. Ultrafiltrates of horse, cow, rabbit, and human serum and bovine amniotic fluid were intermediate in effect and indistinguishable from each other. Not all samples of horse serum were effective and, despite extensive trials, we have not been able to predict without preliminary trial whether a certain lot will be suitable. However, repeated tests on a given lot have given entirely

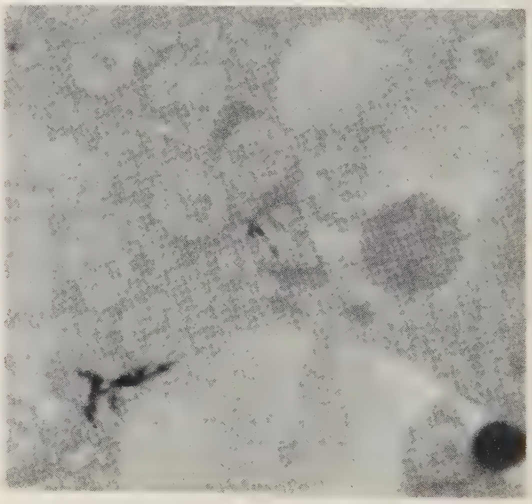

FIG. 4. HeLa cells, human serum medium, 3 days after addition of tubercle bacilli. Not much change from day 2 (Fig. 3), although it is possible to find cells containing considerable bacterial growth.

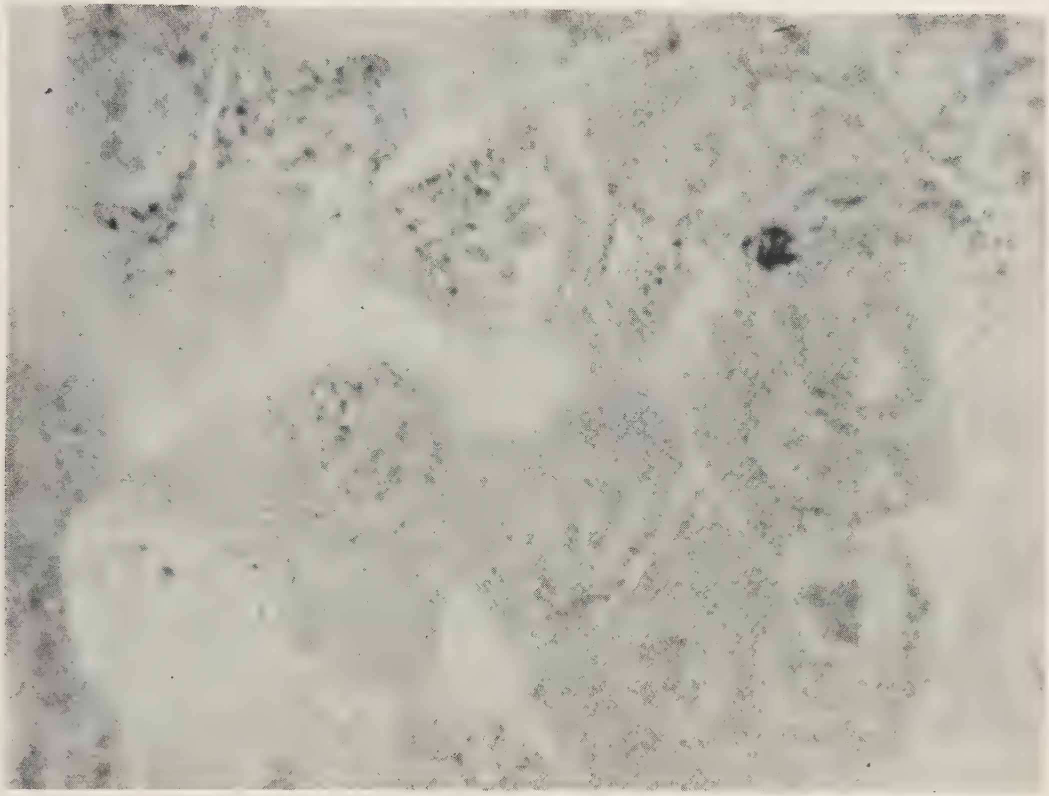

FIG. 5. HeLa cells, horse serum medium, one day after addition of carbon particles and dead tubercle bacilli. Many of the cells have taken the particles and bacilli into the cytoplasm.

uniform results over periods of at least 4-5 months.

Monkey kidney cell cultures in monolayers have shown essentially the same results, that is, favorable horse serum medium is able to increase phagocytosis of living and dead human tubercle bacilli and carbon particles. Luxuriant intracellular growth of the living tubercle bacilli then followed. The results in plasma clot cultures of human foreksin were not so easy to evaluate, perhaps because of the interposition of the clot between cells and culture medium.

The sensitivity of HeLa cell cultures in suitable horse serum culture fluid, as growth medium for small numbers of tubercle bacilli was indicated by several comparisons with bacteriological media, HeLa cell cultures detecting about as many bacilli (H37Rv) as Dubos medium with or without Tween (Difco), and about one-tenth as many as 25% human blood agar(5). It is interesting that the result in HeLa cells could be read in 3-5 days.

Because we were interested primarily in factors affecting intracellular bacillary growth (as a part of a study of tissue culture procedures which might be useful in a study of the leprosy bacillus), we have not attempted to develop methods for the accurate estimation of extracellular bacilli. The number of extracellular bacilli was studied only by smears of the culture fluid, and the strong tendency of the bacilli to clump subjects this method of estimation to considerable error. From the data obtained it is not possible to know whether the total amount of bacillary growth occurring in a culture tube, intra- and extracellularly, was increased as a result of the increased phagocytosis.

Discussion. Certain difficulties are met in interpreting results obtained in tissue culture according to the concepts of infectious processes which are derived mainly from the study of infections in intact animals. Thus it

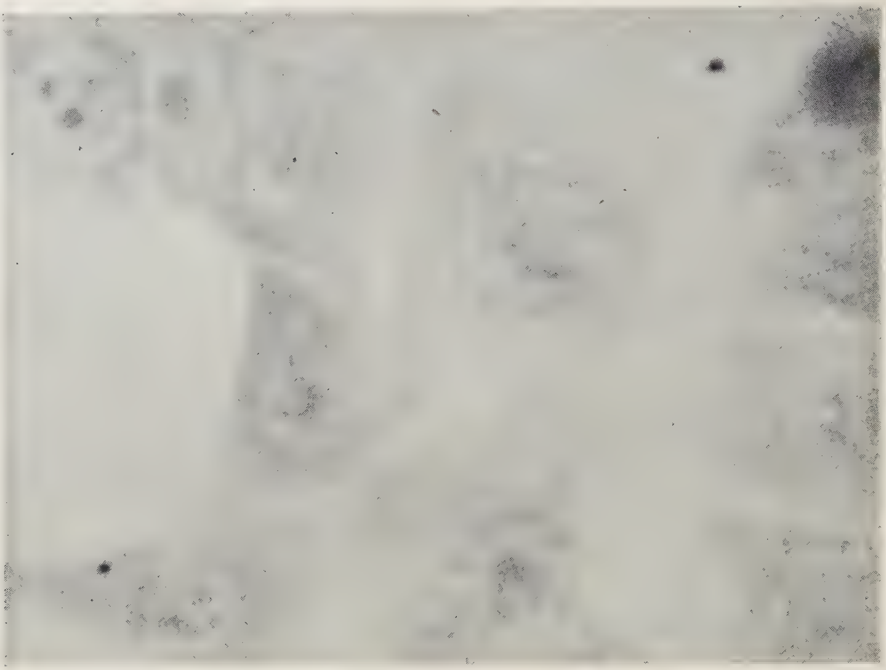

FIG. 6. HeLa cells, human serum medium, one day after addition of carbon particles and dead tubercle bacilli. The intake is considerably less than that in horse serum (Fig. 5), although in the original preparations some small carbon particles may be seen in the cytoplasm of many cells.

is said that a certain cell or tissue culture is susceptible or resistant to a particular infectious agent, because of the practice of considering a mouse or guinea pig as being susceptible or resistant to a given agent. Obviously a more complete statement of experimental conditions is desirable with tissue cultures because of the greater possibility of affecting the multiplication of the infectious agent by changes in culture media, temperature, pH, conditions of inoculation, etc. Even the term "susceptibility" tends to become ambiguous, because in this study, for example, it appears that HeLa cells in our medium are resistant to human tubercle bacilli, yet after the bacilli have gained access to the cytoplasm as a result of use of horse serum media they grow very well in HeLa cells in 40% human serum. A further understanding of the processes by which phagocytosis occurs in a cell such as the HeLa cell may emphasize other semantic obstacles.

Summary. When human tubercle bacilli are added to cultures of HeLa cells, the amount of intracellular growth that ensues depends upon the culture medium on the HeLa cells. If the medium is one containing 40% human serum, 10% chick embryo extract, and 50% BSS, little growth is seen within cells. However, if instead of human serum, certain horse serums are used, tubercle bacilli are rapidly taken up by the cells and luxuriant intracellular growth follows, even if the culture medium is later changed to one containing human serum in place of the horse serum. The mode of action of the horse serum media appears to be due to an increase in phagocytosis, since such media also increase the amount of phagocytosis of dead tubercle bacilli and carbon particles.

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Eosinophil Rhythm in Splenectomized Mice.* (22044)

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As an extension of related work on rats(1), the possible effects of splenectomy upon eosinophil counts and especially upon the 24-hour eosinophil rhythm of mice(2-5) were studied. Twenty-three B_1 males, 2½ months of age, were splenectomized, using Nembutal for anesthesia, while 22 comparable mice were subjected to a sham-operation. Purina Fox Chow and tap water were available to these mice *ad libitum* since weaning and throughout the experiment. For at least 7 days prior to experiment, the mice were singly housed and kept in a room illuminated by artificial light only. A clock-controlled switch turned the lights on at 06:00, and off at 18:00. In order to eliminate effects of previous handling upon the circulating eosinophil level, each group of mice was divided into 2 sub-groups. One subgroup from both the splenectomized group and the sham-operated group was used for sampling of tail blood at one of the 2 times of day chosen for study, namely between 07:00 and 08:30, and between 21:00 and 22:30. The methods employed for eosinophil counts have been described(6).

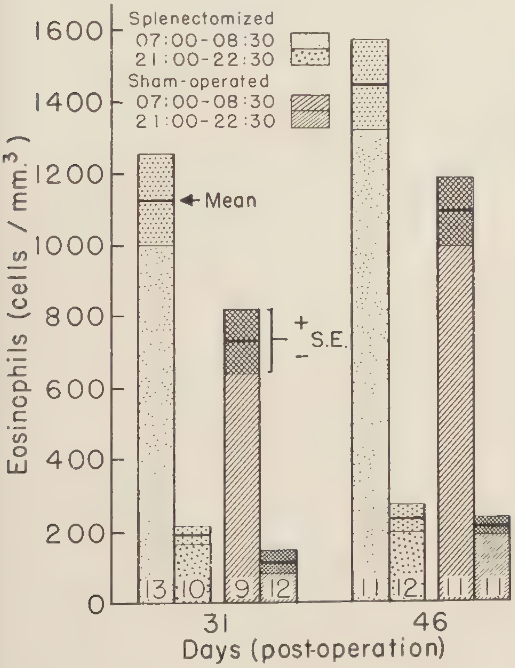

FIG. 1. Eosinophil levels of splenectomized and sham-operated B_1 male mice, at 2 times of day, 31 and 46 days after operation.

TABLE I. Comparison of Eosinophil Levels in Splenectomized Mice and in Sham-Operated Mice, at Several Post-Operative Time Points and at Several Times of Day.

Days after operation	Time of day	Diff. in mean eosinophil count*	t	P
31	7- 8:30	+399	2.41	.026
	21-22:30	+ 87	2.49	.022
46	7- 8:30	+361	2.18	.04
	21-22:30	+ 19	.42	>.60
68	2:50- 4:45	+285	3.12	<.01

* Mean eosinophil count of splenectomized group *minus* the corresponding value of sham-operated group.

The *results,* summarized in Fig. 1, reveal that at 31 and at 46 days after operation, the morning counts were consistently higher than the corresponding evening counts for both the splenectomized group and the sham-operated group ($P<0.001$ in all instances). Clearly, in mice as well as in rats, the spleen may not be necessary for the maintenance of the eosinophil rhythm.

A comparison of counts made at the same time of day in sham-operated mice and splenectomized mice (Fig. 1) also suggests the occurrence of a post-splenectomy eosinophilia, a phenomenon analyzed in Table I. This table includes additional data obtained 68 days after operation, and reveals that the magnitude of post-splenectomy eosinophilia is to some extent a function of the time of day at which the counts were done. But, at the daily times of high eosinophil levels (07:00-08:30),

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the eosinophilia occurring during the second month after splenectomy in mice stands out with unmasked clarity and is statistically significant.

Summary. At several post-operative time points, eosinophil counts were made on tail blood from splenectomized and from sham-operated male B_1 mice, kept under standardized environmental conditions. The occurrence of post-splenectomy eosinophilia was noted. Furthermore, the mean eosinophil counts of all of the groups of mice studied were consistently higher by day than by night. In B_1 mice and rats the presence of the spleen is not essential for the maintenance of eosinophil rhythm.

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Methemoglobinemia Induced by X-Irradiation. (22045)

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Oxidation reactions in aqueous media are commonly enhanced by exposure to ionizing radiation. The iron of the circulating hemoglobin, both reduced hemoglobin and oxyhemoglobin, is in the ferrous or reduced state. Ferric or oxidized hemoglobin occurs in methemoglobin, which amounts normally to 1 to 2% of the total blood pigment(1,2). Fricke and Peterson(3) found that oxyhemoglobin in solution disappears when it is irradiated. They concluded, from spectroscopic observations, that methemoglobin is the principal reaction product. This reaction has been studied to ascertain its usefulness as a quantitative biologic indicator of radiation exposure.

Normal human values. Methemoglobin was determined at various times in 8 apparently healthy laboratory workers, 7 male and one female, by the method of Evelyn and Malloy (4). Total blood pigment was determined after its conversion to methemoglobin by potassium ferricyanide.

The results are summarized in Table I. In our hands, this method was reproducible to ± 0.04% methemoglobin. The mean value of 33 determinations was 1.53 ± 0.30%. The normal methemoglobin level in a given individual was found to vary from time to time; the mean standard deviation of individual variations was 0.33% methemoglobin.

In vitro studies. Hemolyzed washed human red blood cells were mixed with M/60 phosphate buffer, pH = 6.5, in the proportions 1:1, 1:10, 1:20, 1:40, and irradiated with 260 kvp x-rays filtered by 1.0 mm Al and 0.5 mm Cu. The results of a typical experiment are shown in Fig. 1. For doses below 20000 r, the formation of methemoglobin was found to be a linear function of dose. Methemo-

TABLE I. Normal Levels of Methemoglobin (%).

E. R.	J. W.			J. T.		L. W.	W. K.	R. D.	G. S.	S. G.
1.53	.57	2.05	2.06	2.48	1.53	1.77	.53	1.29	1.93	.93
1.62	1.60	2.83	2.74	.82	2.50	1.58	1.82	1.03	1.70	1.23
1.92	1.14	.78	.86	1.06	1.23	2.32	.84	1.22	2.01	1.66
1.69 ± .17	1.66 ± .78			1.60 ± .66		1.89 ± .31	1.06 ± .55	1.18 ± .11	1.88 ± .13	1.27 ± .30

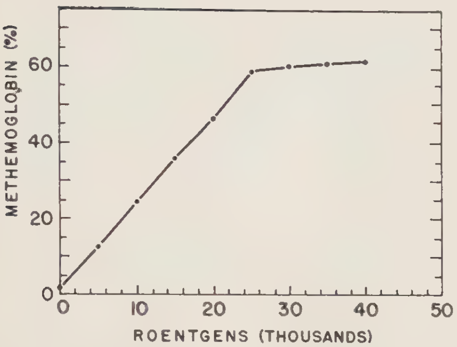

FIG. 1. Methemoglobin formed by irradiation of lysed washed RBC 1:40 in M/60 phosphate buffer, pH = 6.5.

globin was formed in the more dilute solutions at a relatively greater rate than would be expected from the concentration differences.

In vivo experiments. Ten adult male Sprague-Dawley rats were irradiated with 260 kvp x-rays filtered by 1.0 mm Al and 0.5 mm Cu at a rate of 50 r/min. Samples of tail blood for methemoglobin determinations were obtained pre-irradiation, during irradiation after 3000 r, and 6000 r and postirradiation after 1, 3, 5, and 8 hours. The results are depicted in Fig. 2. Variations in the normal methemoglobin values similar to the variations in the human determinations were observed.

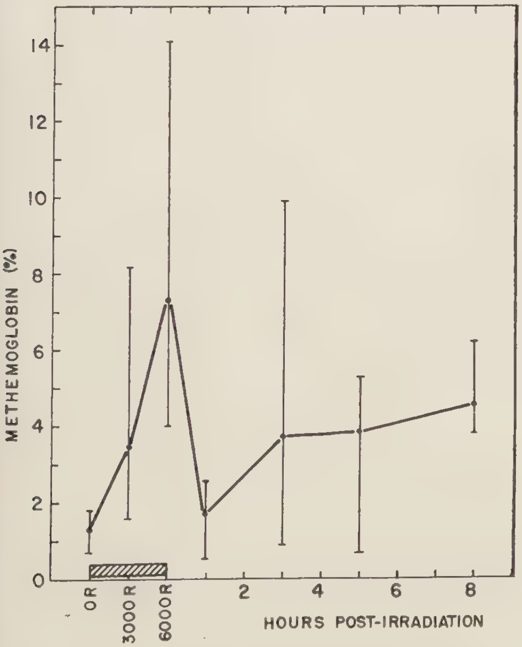

FIG. 2. Methemoglobinemia induced by 260 kvp x-rays in Sprague-Dawley rats with observations in the postirradiation period. Range of values and means are shown.

Discussion. The oxidation of the ferrous iron within the hemoglobin moiety is presumably analogous to the radiation induced oxidation of ferrous salts in aqueous solution. The latter reaction is the basis of one kind of chemical radiation dosimeter(5). The reaction is probably mediated by the oxidative free radicals which are formed upon irradiation of water. In the case of inorganic iron salts, pH and substrate concentration affect the reaction rate. These parameters are held within narrow limits in the circulating blood.

The low one-hour postirradiation methemoglobin level is less than might be expected from reconversion of the methemoglobin in drug induced methemoglobinemias. In experimental drug induced methemoglobinemias the rate of reconversion to hemoglobin was found to be 8-10% per hour(6,7).

Although the increase in methemoglobin was observed to be proportional to radiation exposure in both the intact animal and *in vitro* studies for moderate doses, the usefulness of methemoglobinemia as a quantitative biologic radiation dosimeter appears to have several limitations. Variation was found in the normal baseline levels from individual to individual, and in a given individual from time to time. The secondary postirradiation methemoglobinemia found in the intact animals is a complication. Appreciable methemoglobinemia is produced only after exposures in the lethal and supralethal ranges.

Summary. Methemoglobin forms in aqueous solutions of hemoglobin and in intact rats after exposure to ionizing radiation. For moderate doses, the degree of methemoglobinemia is a linear function of dose. In the sub-lethal dose range, the methemoglobinemia is comparable to the random variation in normal values. A secondary methemoglobinemia was observed in rats 3 to 8 hours postirradiation.

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Relation of Sex and Age to Resistance of Mice to Experimental Histoplasma Infections.* (22046)

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Preliminary studies from this laboratory indicated that age affected the resistance of white Swiss male mice to experimental histoplasmosis(1). Campbell and Saslaw(2) using 14- to 17-g white mice noted a greater mortality in males. Salvin(3) reported no sex differences in mortality of 35-day-old mice. The purpose of this report is to elucidate further the sex and maturity factors relative to histoplasma infections in mice.

Materials and methods. Five-week-old white Swiss mice of both sexes were obtained at one- or 2-week intervals and kept until there were groups representing these age intervals from 5 through 23 weeks. The numbers of mice used in each group varied according to availability. The mice were inoculated intraperitoneally with 23 or 35 million yeast phase *H. capsulatum* organisms in 0.5 ml of 5% hog gastric mucin. All mice were weighed on the day of inoculation, and at weekly intervals. The mice were observed for a 30-day period and necropsy performed on those dying during this interval.

Results. Table I summarizes the results of these studies. Column A in Table I demonstrates that 10- to 15-week-old male mice receiving 23 million organisms showed increased resistance to fatal infection as compared to those 5 and 9 weeks old. A further increase in resistance was noted in those 16 weeks or older. With the 35 million dose (column C) the increased resistance was first noted at 17 weeks of age. The parallel studies in female mice with the lower dosage (column B) showed a similar increase in resistance at 8 weeks as compared to 10 weeks in males and a further increase in survival rate after 15 to 16 weeks of age. With the 35 million dose (column D) the 2 periods of comparative increased resistance were noted at 9 and 17 weeks in females and males, respectively. In comparing the death rates in both sexes receiving 23 million organisms the 8-week-old male mice showed 19 of 22 deaths (86.5%) as compared to 14 of 25 female deaths (56%); in the 9-week-old group, 9 of 10 males (90%), and 7 of 11 females (63.3%) died. Thereafter the incidence was similar except at 15 weeks when 8 of 12 males, as compared to 4 of 10 females, died. In those mice receiving 35 million organisms the differences in resistance of sexes became apparent in the 9-week-old mice where 15 of 16 males (93.7%) as compared to only 9 of 15 females (60%) died. This difference became less apparent by the 13th week and no appreciable variation was noted thereafter. Combining the total mortalities under both sex groups the 8- to 14-week period represented the time interval when apparent sex differences in susceptibility occurred (Table II). As can be seen in Table II, when all of the age groups were combined no significant difference between sexes was noted following inoculation with 23 million organisms, since 174 of 337 females (51.6%) died as compared to 183 of 331 males (55.3%). However, with the 35 million dose, the males were significantly more susceptible in that 105 of 145 males (72.5%)

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* This investigation was supported in part by research grant from the National Institute of Health.

TABLE I. Effect of Age on Resistance of White Swiss Mice to Histoplasmosis.

Challenge dose, 23 million					Challenge dose, 35 million				
	Deaths					Deaths			
	A ♂		B ♀			C ♂		D ♀	
Age (wk)	No.	%	No.	%	Age (wk)	No.	%	No.	%
5	10/10*	100.0	10/10	100.0	5	15/15	100.0	15/15	100.0
6	31/32	96.8	32/33	96.9					
7	10/10	100.0	10/10	100.0	7	15/15	100.0	14/15	93.3
8	19/22	86.4	14/25	56.0					
9	9/10	90.0	7/11	63.3	9	15/16	93.7	9/15	60.0
10	13/25	52.0	13/26	50.0					
11	6/10	60.0	6/10	60.0	11	13/15	86.6	10/15	66.7
12	13/24	54.2	13/27	48.1					
13	8/11	72.7	6/10	60.0	13	10/12	80.0	11/16	68.7
14	16/26	61.5	14/23	60.8					
15	8/12	66.7	4/10	40.0	15	12/15	80.0	11/15	73.3
16	8/23	34.8	8/25	32.0					
17	3/9	33.3	4/10	40.0	17	7/15	46.7	6/15	40.0
18	6/27	22.2	8/26	30.7					
19	3/10	30.0	4/9	44.0	19	5/14	35.7	4/14	28.0
20	5/24	20.8	7/25	28.0					
21	3/10	30.0	3/10	30.0	21	7/15	46.7	5/14	35.7
22	8/26	30.7	7/27	25.9					
23	4/10	40.0	4/10	40.0	23	6/14	42.8	7/15	46.7

* Numerator = No. of deaths. Denominator = Total No. inoculated.

died as compared to 92 of 149 females (61.7%). As can be seen in Table III, the increase in resistance was not merely a difference of weight. For instance, while the weight of male animals from 5-9 weeks of age increased 4.4 g (12.9 to 17.3 g) there was no significant increase in resistance, but between the 9th and 10th week the fatality dropped from 94.4 to 59.2% while the weight increased only 0.2 g.

Discussion. The data presented in these studies suggest that age, sex and dosage affect the susceptibility of white Swiss mice to experimental histoplasma infections. Increased resistance became apparent by the 8th and 10th weeks, respectively, in females and males when 23 million organisms were inoculated. With more severe challenge (35 million organisms) increased resistance was noted at 9 weeks of age in females; the males showed equal susceptibility through 15 weeks, but increased resistance at 17 weeks. These latter results differ slightly from those previously reported by us in which the period of increased resistance in males was noted at 14 weeks of age(1). This difference may be attributed to the fact that a more careful definition of age was made in this study in that all mice were originally obtained at 5 weeks of age, and then held until the desired age group was obtained. In the previous study the specified age groups were ordered from the animal farm which could have resulted in more variation in comparative ages. Thus, it appears that females become more resistant at a slightly earlier age (8 weeks, as compared to 10 in males) with 23 million organisms as inoculum, but this difference was more marked after the 35 million dose, in that females showed significantly greater survival as early as 9 weeks of age, as

TABLE II. Comparison of Resistance of Male and Female Mice of Same Age Groups.

		5-7-wk mice		8-14-wk mice		15-23-wk mice		Total	
		Deaths							
Dose (million)	Sex	No.	%	No.	%	No.	%	No.	%
23	♀	52/53*	98.1	73/132	55.3	49/152	32.2	174/337	51.6
23	♂	51/52	98.0	84/128	65.6	48/151	31.7	183/331	55.3
35	♀	29/30	96.7	30/ 46	65.2	33/ 73	45.2	92/149	61.7
35	♂	30/30	100	38/ 43	88.4	32/ 72	51.4	105/145	72.5

* No. of deaths/No. of mice infected.

TABLE III. Comparison of Weights with Age and Resistance of Mice.*

Age (wk)	Sex	Variations in avg wt (g) of age groups	Increase in avg wt (g) of age groups	Avg % fatalities of group
5- 9	♂	12.9-17.3	4.4	94.4
10		17.3-17.5	.2	52.0
11-15		17.5-21.3	3.8	59.2
16		21.3-21.4	.1	34.8
17-23		21.4-23.5	2.1	28.7
5- 7	♀	12.6-15.3	2.7	98.1
8		15.3-15.7	.4	56.0
9-14		15.7-19.0	3.3	55.3
15		19.0-19.3	.3	40.0
16-23		19.3-22.4	3.1	32.2

* Inoculated with 23 million yeast phase *H. capsulatum* in 0.5 ml mucin.

compared to 17 weeks in males. Between 8 to 14 weeks of age females appeared to be especially more resistant than males. This observation can probably be attributed to the fact that female mice mature more rapidly than males(4). Thus, before and after full development of sexual maturity the differences in susceptibility were not appreciable. These results are also comparable to those observed by Theiler(5) in which the morbidity and mortality rates in experimental encephalomyelitis of mice were lower in mice over 12 weeks of age. The recognition of such differences in susceptibility in different age and sex groups can assist in the evaluation of data in a variety of studies employing the mouse as the experimental animal.

Conclusions. Age, sex, and dose are important factors in comparative susceptibility of mice to fatal histoplasma infections. Both sexes may show lower mortality ratios with increasing age. Females develop resistance earlier than males. This difference is most apparent between 8 to 14 weeks.

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Laboratory and Clinical Observations on Mecamylamine as a Hypotensive Agent.* (22047)

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Currently available ganglionic blocking agents used for the treatment of hypertension produce an inconstant day to day reduction in blood pressure largely due to incomplete and variable absorption of the quaternary ammonium compounds (bisammonium) from the intestinal tract. Mecamylamine, a secondary amine† is the first known ganglionic blocking agent which is completely absorbed after oral administration. The hemodynamic effects have been studied both in the laboratory as well as in patients with hypertension.

Methods and materials. Laboratory observations: Observations on the blood pressure response and on ganglionic blockade were made on 29 dogs anesthetized with 30 mg/kg of pentobarbital. The dogs were divided into 2 groups. Group I consisted of 12 dogs on which observations were made relative to doses which effectively blocked the autonomic ganglia and consequently reduced the blood pressure. Group II consisted of 17 dogs on which observations on the effect on renal hemodynamics and the excretion rates of water and electrolytes were made. *Group I.* Arterial blood pressure was measured by direct intra-arterial manometry in these ani-

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† 3-Methylaminoisocamphane hydrochloride. Available as Inversine by Sharp & Dome, Division of Merck & Co., West Point, Pa.

mals. Following suitable control observations, dosage increments of .05, .5, 1 and 2 mg/kg of mecamylamine were given intravenously over a 5-minute period. Subsequently, observations on blood pressure were made for the ensuing 5 hours. During this period, the blood pressure responses to electrical stimulation of the peripheral end of the severed vagus and to carotid occlusion were made at regular intervals. At one hour intervals, norepinephrine was infused at the same rate as required to produce a rise in mean blood pressure of 20 mm Hg during the control period. This was done as a test for adrenergic blockade. *Group II.* (*Renal studies*). Seventeen female dogs ranging in weight from 9 to 15 kg were used. They were anesthetized with pentobarbital, 30 mg/kg body weight, given intravenously. Creatinine was used to measure glomerular filtration rate (GFR) and p-aminohippurate (PAH) for renal plasma flow (RPF). Sodium and potassium determinations were made using a Beckman flame photometer for analysis. Arterial blood, collected in heparinized syringes, was used for chemical analysis. Analytical methods and procedures have been described previously(1). Mean blood pressure (MBP) was measured by a damped mercury manometer connected through a manifold to an indwelling arterial needle.

Clinical observations. The clinical observations were made on 2 groups of patients with moderately severe to severe hypertension. Group I consisted of 12 patients who were observed in detail and in whom observations were made on single dosage responses giving the drug no more frequently than once every other day. Group II consisted of 50 patients who received the mecamylamine as therapy for their hypertension. *Group I.* The 12 patients in Group I consisted of hospitalized patients with moderate to severe hypertension. After suitable control observations were made on the day of the test, 5 mg of mecamylamine were given orally at 8 o'clock in the morning. Observations on blood pressure were again made in the supine and upright positions every 15 minutes for the first 3 hours and then every half hour for the remainder of the day. If an adequate reduction in blood pressure was not obtained, a larger dose was given 2 or more days later and the procedure repeated. The dose was thus increased in 5 mg increments until an effective reduction in blood pressure‡ was obtained for each patient. Subsequently, 5 of the patients were given the same dose (effective dose) by the parenteral route in order to compare the response by the 2 different routes of administration. *Group II.* (Out-patients with Hypertension): All patients in this group had received 1 mg of reserpine a day for several months before initiation of the current study. This was not altered. None of the patients had become normotensive and control observations for the current study were made 2 months or more after stabilization on reserpine. The reserpine was continued because it reduces the pulse rate and blocks the reflex orthostatic tachycardia which is frequently observed with patients given ganglionic blocking agents.

This study consisted of a group of 50 patients with moderate to severe essential hypertension treated as out-patients in the Hypertensive Clinic. All patients had control blood pressures, as measured in both the supine and upright positions, of 150/100 mm Hg or greater. Controls were established by taking the average of a minimum of 4 separate blood pressure and pulse rate determinations in the supine and upright positions while the patients received only placebo. The post drug observations (2 months or more) are the averages of all blood pressures and pulse rates taken weekly in both the supine and upright positions after the therapeutic dose of mecamylamine for each patient had been established (*i.e.,* after the titration period during which time blood pressure and drug dosage were being stabilized). Sixteen of the group were males and 34 were females. Five were white and 45 negro. Average age was 52 years, with a range of 34 to 69 years. The severity of the disease in this group is best indicated by a high incidence of complications and by the fact that 41 (82%) had the retinal

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‡ An attempt was made to reduce the standing blood pressure to a level below 150/100 without prohibitive side reactions.

TABLE I. Mean Blood Pressure* Response to Graded Doses of Mecamylamine Given Intravenously to Dogs.

		Time after drug in min.									Dose,
Dog No.	Control	5	10	15	20	30	45	90	120	300	mg/kg
1	128	96	90	84	80	60	55	50	44	44	2
2	132	110	100	106	104	100	102	100	102	104	2
3	142	98	100	104	116	102	104	110	128	136	2
4	164	154	144	128	126	128	134	142	146	146	.5
5	115	108	106	100	90	84	70	65	70	88	.5
6	168	142	135	136	138	132	128	128	126	130	.5
7	140	132	128	116	114	96	94	94	112	104	1
8	128	120	100	85	82	80	90	80	95	95	1
9	110	65	65	60	50	54	60	58	76	88	1
10	138	140	140	138	130	134	130	126	128	128	.05
11	138	136	138	138	140	140	144	146	148	154	.05
12	160	160	158	156	164	160	162	158	160	160	.05

* Direct intra-arterial manometry.

changes of hypertensive vascular disease; 7 of these had retinal hemorrhages. The initial dose of mecamylamine was 5 mg given orally twice a day (after breakfast and supper). This dose was empirically chosen as one which would not produce excessive hypotension or side reactions. However, in some cases, this dose resulted in marked orthostatic hypotension and numerous side reactions. A more suitable starting dose would be 2.5 mg twice a day. In our experience, this dose rarely produces side reactions or excessive hypotension. The initial dose was gradually increased in a stepwise fashion until an adequate blood pressure response was obtained. To minimize the orthostatic side reactions from excessive hypotension, the blood pressures in the upright position were the guide to the weekly changes in dosage. In addition to the blood pressure observations, clinical manifestations of excessive hypotension such as dizziness on standing and weakness were considered when regulating the dosage schedule.

Results. General Laboratory Observations (*Group I*): A consistent blood pressure effect in dogs was not observed with doses of less than 0.1 mg/kg. Doses in excess of 0.5 mg/kg did not cause much greater reduction in blood pressure than the 0.5 mg/kg dose but the period of the depressor response was not as long with smaller doses as with the larger ones. Likewise, the onset of action was somewhat more rapid with larger doses. Following administration of 0.5 mg/kg or more, this delay period was 5 to 10 minutes before the blood pressure decreased, and maximum depression was observed after 30 to 45 minutes (Table I). Blockade of the depressor response to vagal stimulation was consistent at the 1 mg/kg level. Blockade of the carotid pressor reflex usually occurred at a dose of 0.5 mg/kg. When maximum reduction in blood pressure occurred, there was no evidence of adrenergic blockade. In fact, the dose of norepinephrine required to produce a rise in mean blood pressure of 20 mm Hg during the control period was usually excessive after the administration of mecamylamine, apparently a result of ganglionic blockade of vasopressor inhibitory reflexes which are mediated via the parasympathetics. These observations indicate that the vasodepressor effect is not due to adrenergic blockade. Ganglionic blockade (partial) is present, but an additional central component has not been ruled out.

Observations on Renal Hemodynamics (Group II): Doses of mecamylamine of 0.2 and 0.5 mg (approximately .03 to .05 mg/kg) had no effect on blood pressure, renal hemodynamics, or on water and electrolyte excretion. Doses of 0.5 to 2.0 mg/kg produced a significant reduction in blood pressure ($p < 0.001$) which was more prolonged after the 1 and 2 mg/kg doses than after the .5 mg/kg dose. However, the initial reduction in blood pressure appeared to be nearly the maximum response with the 0.5 mg dose and was not increased by giving a larger dose. The initial reduction in blood pressure was not associated with depression in glomerular filtration rate or in renal blood flow during the first 30 minutes after drug administration. Renal vasodilata-

TABLE IIA. Effect of Mecamylamine on Renal Hemodynamics.

Dog No.	Mean blood pressure, mm Hg				Glomerular filtration rate, ml/min.				Renal blood flow, ml/min.				Renal vascular resistance*				Dog wt, kg	Dose
	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3		
Sub-group A																		mg
1	126	131	140	147	38	27	34	38	177	216	216	196	.71	.61	.65	.75	13.0	.2 (Total)
2	158	157	104	115	35	35	31	30	198	194	222	218	.80	.81	.47	.53	16.5	.2 "
3	132	132	126	142	37	33	41	35	202	200	234	198	.65	.66	.54	.72	11.0	.2 "
Mean	139	140	123	135	37	32	35	34	192	203	224	204	.72	.69	.55	.67	13.5	.2
Sub-group B																		
4	163	159	163	160	62	57	56	66	318	363	402	342	.51	.44	.41	.47	18.0	.5 (Total)
5	135	136	139	152	64	65	64	66	437	452	354	289	.31	.30	.39	.53	18.8	.5 "
6	138	137	135	128	50	55	50	44	320	317	260	185	.43	.43	.52	.69	18.0	.5 "
Mean	145	144	146	147	59	59	57	59	358	377	339	272	.42	.39	.44	.56	18.3	.5
Sub-group C																		mg/kg
7	139	121	113	111	45	53	40	37	251	269	195	170	.55	.45	.58	.65	15.0	.5
8	123	88	90	97	49	47	40	44	328	354	316	250	.38	.25	.28	.39	18.5	.5
9	63	43	40	64	30	30	30	31	134	132	121	106	.47	.33	.33	.60	12.0	.5
10	138	123	95	107	50	50	47	39	266	292	313	202	.52	.42	.30	.53	14.0	.5
11	103	60	56	89	49	44	44	34	192	180	219	120	.54	.33	.26	.74	20.0	.5
12	116	102	66	85	47	47	33	25	283	276	192	157	.41	.37	.34	.54	12.5	1.0
13	162	132	137	144	37	35	33	33	204	169	135	132	.79	.78	1.01	1.09	12.0	1.0
14	159	136	138	129	83	87	74	55	495	562	459	317	.32	.24	.30	.41	22.0	1.0
15†	123	89	47	45	44	38	9	15	284	306	42	208	.43	.29	1.12	.22	11.0	1.0
16	119	106	105	101	27	26	25	26	185	146	112	116	.64	.73	.94	.87	13.0	2.0
17	141	102	113	132	48	44	42	41	313	281	233	200	.45	.36	.48	.66	15.0	2.0
Mean	126	100	91	100	46	46	38	35	267	270	212	180	.50	.41	.54	.61	15.0	1.0
% of control		79	72	79		100	83	76		101	79	67		82	108	122		
P value‡ (<)		.001	.001	.001		NS	.05	.01		NS	.05	.001		.01	NS	.05		

C = Avg of 3-10 min. periods; control. D_1 = Immediately after drug administration (avg of 2-10 min. periods). D_2 = One hr after drug. D_3 = 3 hr after drug.

* Renal vascular resistance $= \frac{\text{Mean blood pressure}}{\text{Renal blood flow}}$.

† Dog died 8 hr later.

‡ $t = \bar{x}\sqrt{\frac{n(n-1)}{Sx_2}}$ (Sub-group C). Statistical analysis by R. A. Seibert, Ph.D.

TABLE IIB. Effect of Mecamylamine on Water and Electrolyte Excretion.

Dog No.	Urine vol, cc/min.				Sodium excretion, meq/min.				Potassium excretion, meq/min.				Hematocrit			
	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3	C	D_1	D_2	D_3
Sub-group A																
1	.6	1.9	1.1	.9	45	75	42	62	15	18	30	38	39	42	45	43
2	.5	.3	.7	.7	18	13	14	12	22	18	16	19	38	38	45	49
3	1.0	1.0	2.1	2.0	103	85	223	81	26	26	38	33	39	40	41	44
Mean	.7	1.1	1.3	1.2	55	58	93	52	21	21	28	30	39	40	44	45
Sub-group B																
4	1.0	1.6	1.8	5.5	45	68	62	60	24	32	42	62	32	35	35	34
5	1.8	2.5	2.2	1.7	285	382	302	182	43	64	64	70	41	40	41	43
6	3.7	2.7	1.6	1.6	269	272	170	175	30	29	23	27	35	35	35	33
Mean	2.2	2.3	1.9	2.9	200	241	178	139	32	42	43	53	36	37	37	37
Sub-group C																
7	3.0	1.8	2.4	1.5	257	135	84	47	43	49	44	40	37	36	36	39
8	1.2	1.2	2.1	2.2	190	152	183	166	42	38	56	51	39	39	37	38
9	.5	.6	.6	.9	25	37	39	44	19	22	24	31	41	38	34	36
10	1.5	1.8	1.4	1.2	178	215	137	75	28	41	38	36	39	39	38	35
11	7.1	5.9	6.7	2.2	722	639	678	188	97	111	110	61	41	39	36	35
12	.5	.5	.4	.6	18	37	15	24	27	41	16	24	40	41	40	40
13	1.0	1.1	1.3	2.1	47	61	62	80	22	23	21	19	51	48	45	43
14	3.6	7.3	5.7	4.1	291	403	173	103	74	73	57	58	35	35	36	40
15	1.0	.7	.3	.4	87	58	6	9	40	25	1	5	43	47	45	47
16	2.1	2.3	1.2	1.6	123	186	93	136	29	39	30	45	35	35	32	32
17	1.1	.7	1.4	1.5	50	92	87	122	41	42	37	38	39	38	37	37
Mean	2.1	2.2	2.1	1.7	181	183	142	90	42	46	39	37	40	40	38	38
% of control		105	100	81		101	78	50		110	93	88		100	95	95
P value* (<)		NS	NS	NS		NS	.10	.10		.20	NS	NS		NS	.05	.20

C = Control (avg of 3-10 min. periods). D_1 = First 20 min. after drug administration (avg of 2-10 min. periods). D_2 = One hr after drug administration (avg of 2-10 min. periods). D_3 = 3 hr after drug administration (avg of 2-10 min. periods).

* $t = \bar{x}\sqrt{\frac{n(n-1)}{Sx^2}}$ (Sub-group C).

tion (decreased renal vascular resistance), was adequate ($p<0.01$) to compensate for the reduction in blood pressure. However, with prolonged hypotension, there was significant reduction in glomerular filtration rate and in renal blood flow ($p<0.05$). This was probably enhanced by the anesthesia and the fact that the ganglionic blockade persisted throughout the period of observation in contrast to hexamethonium, which is active for a much shorter period of time(2,3). The reduction in glomerular filtration rate was not accompanied by alterations in water and electrolyte excretion.

Clinical observations on acute responses to single doses (Group I): The average single dose which would reduce the blood pressure to approximately normotensive levels in the upright position when administered orally was 19 mg but the range was 5 to 40 mg. The duration of action varied from 12 to 48 hours with an average of 18 hours. There was a latent period of 20 to 70 minutes after parenteral administration of mecamylamine and 30 minutes to 2 hours after oral administration. Table III shows that the final response to equal oral and parenteral dosages was about the same. After an effective dose was given, increasing the dose had very little additional effect on the supine blood pressure but markedly enhanced and prolonged the upright pressure response.

Out-patient treatment of hypertension (Group II): For purposes of analysis, the 50 patients have been divided into 2 sub-groups on the basis of their upright control diastolic blood pressure. Sub-group I consisted of 9 patients with less severe hypertension, *i.e.*, their control upright diastolic blood pressure was greater than 100 but less than 120 mm Hg.

TABLE III. Summary of Data for Single Dosage Response to Mecamylamine in 12 Patients with Hypertension.

Patient		Control blood pressure				Blood pressure max response				Onset blood pressure reduction (min.)	Duration blood pressure reduction (hr)	Dose (mg)*	Route	Side effects	Renal disease	Ocular fundi†	Age
		Supine		Upright		Supine		Upright									
		S	D	S	D	S	D	S	D								
D.D.	A	188	122	182	120	132	78	102	72	60	14	10	Oral	0	0	III	59
J.H.	A	220	140	200	130	200	130	140	100	90	16	15	”	0	+++	II	47
B.N.	A	230	110	230	110	150	100	70	50	90	24	5	”	+++‡	+++	II	61
F.H.	A	170	110	160	100	140	100	140	100	90	24	5	”	0	0	I	59
C.A.	A	200	120	190	110	180	110	110	80	30	14	20	”	0	++	II	56
P.M.	A	230	130	210	120	188	108	138	90	90	12	40	”	++‡	+++	III	58
J.R.	A	188	122	190	120	160	110	120	70	90	16	25	”	++‡	+++	III	66
T.T.	A	222	162	228	158	178	109	122	78	120	12	30	”	0	+++	III	44
	B	230	158	226	156	170	107	128	72	70	14	30	Intramusc.	0	—	—	—
T.R.	A	198	138	192	128	132	76	112	82	30	48	10	Oral	0	0	II	38
	B	188	140	190	120	140	72	118	78	60	48	10	Intramusc.	0	—	—	—
K.C.	A	232	144	218	148	190	118	138	108	90	12	40	Oral	0	+++	IV	34
	B	250	154	238	150	178	112	146	104	60	16	40	Intramusc.	0	—	—	—
J.M.	A	198	110	190	115	140	80	120	78	30	12	15	Oral	0	++	II	60
	B	186	112	188	112	146	88	116	76	20	15	15	Intramusc.	0	—	—	—
L.T.	A	236	138	225	134	190	106	128	88	120	18	10	Oral	N & V	+	II	42
	B	236	136	256	137	225	110	160	100	30	16	10	Intramusc.	N & V	—	—	—

* This dose (established by titration in 5 mg increments) would repeatedly reduce the blood pressure to normotensive levels while standing.

† Keith-Barker-Wagener classification.

‡ Side effects consisted primarily of marked weakness and dizziness (BN) when standing.

S = Systolic blood pressure. D = Diastolic blood pressure.

A = Observations after oral administration. B = Observations after parenteral administration using same dose as A.

TABLE IV. Comparison of Blood Pressure Response—Mecamylamine, Hexamethonium and Pentolinium.

	+ Rauwolfia +		+			
	Mecamylamine		Hexamethonium		Pentolinium	
	No.	%	No.	%	No.	%
No. patients treated	50	100	75	100	75	100
Responsive	46	92	57	76	59	79
Normotensive	12	24	28	37	25	33
Unresponsive	4	8	18	24	16	21
Avg daily dose of responders	17 mg		2307 mg		341 mg	

Sub-group II consisted of 41 patients with more severe hypertension (*i.e.*, control upright diastolic pressures greater than 120 mm Hg). The criterion for response was a fall in the mean control blood pressure of 20 mm Hg or more, or a return to normotensive levels (<150/100 mm Hg) in the upright position. The overall response rate was 92% and 24% of the patients became normotensive on a total daily dose of 17 mg of mecamylamine. Of the 9 patients with control diastolic pressures less than 120 mm Hg but greater than 100 mm Hg (Sub-group I), 7 (78%) responded while 2 (22%) of these became normotensive on an average dose of 13 mg of mecamylamine daily. In sub-group II (control diastolic blood pressures greater than 120 mm Hg in the upright position), 39 (95%) of the 41 patients were responsive and 10 (24%) returned to normotensive levels on an average daily dose of 19 mg of mecamylamine. The fall in blood pressure was greatest in the upright position; however, there was also a significant reduction in the supine blood pressure.

It is difficult to evaluate the beneficial effects of blood pressure reduction due to the short period of treatment with mecamylamine. However, the most outstanding symptomatic improvements were relief of headaches and angina pectoris which were present in two-thirds of the patients prior to therapy. About one-half of the patients with congestive heart failure showed symptoms of improvement during this short course of treatment.

The most annoying side effects with mecamylamine were constipation, weakness and dizziness. Constipation was adequately controlled by the use of cathartics such as milk of magnesia, cascara, and mineral oil. Although constipation was not marked in most cases, it is a serious side effect and should be treated actively to prevent fecal impaction and ileus. After continued therapy for several weeks, this side effect either lessens or disappears in most patients. Weakness was present in 38% of the group. In most patients, this was not marked and was hard to evaluate. Dizziness was present in 40% at some time during the course of therapy. In some cases, this was due to excessive orthostatic hypotension although syncope was not observed. Dizziness was marked in 3 patients and in these it was probably due to over zealous treatment. Nausea was present at some time during the course of therapy in 10 (20%) of the patients but only 3 (6%) vomited. Several other less serious side effects such as blurred vision and dry mouth were observed in a number of the patients. Comparison of the side effects of therapy with mecamylamine and 2 other ganglionic blocking agents reveals that the major side effects of ganglionic blockade are qualitatively comparable with the 3 drugs.

Summary and conclusions. Mecamylamine produced the same cardiovascular and renal hemodynamic effects on dogs as have been previously observed with such autonomic blocking agents as hexamethonium and pentolinium. When given to patients with hypertension, it produced a consistent reduction in blood pressure which was most marked in the upright position. The effect was about equivalent when given orally as when given parenterally, the average daily dose being approximately 19 mg. The onset of action ranged from ½ to 2 hours, and lasted 12 to 48 hours. The response was quite variable from patient to patient, but within the same patient, it was reproducible, thus minimizing one of the chief objections to ganglionic blocking agents for the treatment of hypertension.

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An Isolated Human Voluntary Muscle Preparation.* (22048)

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The use of isolated mammalian and amphibian nerve-muscle preparations for the study of drug and ion interactions at the motor end-plate is well known. The object of this communication is to describe a method of utilizing isolated human muscle obtained from surgical operations for similar study. Fasciculi from various muscle groups have been tried, but so far the human intercostal, obtained at thoracotomies, has been used most often because of its small discrete fasciculi, short fibers, availability, and accessibility.

Method. After the muscle specimen was removed, a suitable fasciculus was dissected out and placed in a vial containing Krebs' solution, which had been saturated with 95% O_2 and 5% CO_2. The vial was closed and initially packed in chipped ice during the time it was transported from the hospital to the laboratory. Subsequently it was ascertained that chilling of the muscle was unnecessary, and this practice has been discontinued. Each fasciculus was about 0.5-1.0 mm thick and 10-15 mm long. It was suspended in a bath of Krebs' solution between 2 platinum electrodes as previously described(1). Tracings were usually about 20-30 mm in height. Because the nerve supply to the muscle cannot ordinarily be isolated, an attempt was made to stimulate the intramuscular nerves without at the same time stimulating the muscle fibres themselves by using a square wave of very short duration. The square wave stimulator was triggered by one of a pair of rocking mercury switches; the other mercury switch was used to stimulate the muscle fibres directly as before(1), and the switches were rocked to provide alternate direct and indirect stimuli at 10-second intervals.

The electrodes used to stimulate the intramuscular nerves were placed about 5 mm apart on either side of the muscle rather than longitudinally since Rushton(2) has shown that transverse current preferentially affects intramuscular nerves. To the same end 10%, rather than 5%, carbon dioxide in oxygen was used, since evidence has been presented(3) that conditions which lower the pH *in vitro* reduce the chronaxie of nerve relative to that of muscle in the human subject. In our experience the use of 10% carbon dioxide did favor the stimulation of intramuscular nerves selectively. It has been determined that a square-wave pulse of 0.01-0.07 millisecond duration and 5-10 volts (as measured across the electrode by an oscilloscope) will stimulate only the nerve endings in this preparation, and the contractions elicited by the indirect stimuli are equal to or slightly less than those from direct stimuli. Furthermore, contractions from indirect stimuli are blocked completely by 1-1.5 μg/ml of decamethonium bromide or 2.5-3.0 μg/ml of d-tubocurarine chloride.

There was some evidence that the action of C10 on this muscle was biphasic as previously reported for the rabbit lumbrical(1).

Summary. A preparation of human voluntary muscle suitable for studies on neuromuscular transmission *in vitro* is described and some of the actions of curare and of decamethonium on it are recorded.

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Effect of Amniotic Fluid on Cortisone Inhibition of Growth. (22049)

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Normal growth under certain conditions is inhibited by systemic cortisone. One outstanding example of this biologic property of cortisone is its ability to inhibit normal wound healing(1,2). The local applications of beef amniotic fluid have been observed to reverse completely this systemic action of cortisone on healing wounds(3). Accordingly it seemed advisable to determine the effect of beef amniotic fluid upon other growing cellular systems. For this purpose the newly hatched chick was selected as a model.

Methods. Newly hatched White Rock chicks, obtained from a local hatchery, were fed Purina chick starter and water *ad lib.*, while being kept in a chick brooder incubator in the laboratory. The chicks were divided into the following groups: (a) 37 chicks were allowed to grow normally for 18 days; (b) 36 chicks received 5 mg of Cortisone (Merck & Co.) subcutaneously daily for 18 days;[†] (c) 27 chicks received daily subcutaneous injections of beef amniotic fluid for 18 days. The dose was increased from 5 cc to 10 cc per day as the chicks grew; (d) 35 chicks received 5 mg of cortisone in addition to 5 to 10 cc of beef amniotic fluid per day for 18 days. All chicks were weighed at the beginning of the experiment and at 18 days. Amniotic fluid was not given on the 18th day.

Results. The average weight gain of each chick per group is illustrated in Table I. Fig. 1 shows a photograph of three 18-day-old chicks. The chick on the left received only cortisone. The chick in the middle received cortisone plus beef amniotic fluid. The chick on the right is a normal 18-day-old chick. This study indicates that the chicks receiving beef amniotic fluid in addition to cortisone are larger and better developed than their mates receiving cortisone alone; although not the equal of their normal mates. It is of interest to note that beef amniotic fluid administered alone did not promote or accentuate normal growth. In fact, the chicks of group (c) show less average weight gain than normal.

TABLE I.

Group	No. of chicks	Avg wt gain in 0-18 days (g)
A. Normal growth	37	136.2
B. Cortisone treated	36	28.9
C. Amniotic fluid treated	27	91.3
D. Cortisone & amniotic fluid	35	49.9

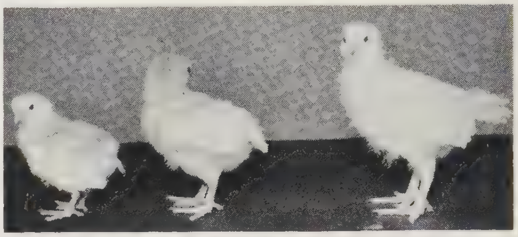

FIG. 1. Three 18-day-old chicks. Left chick given 5 mg cortisone daily, middle chick given cortisone plus amniotic fluid, right chick represents normal growth.

Beef amniotic fluid is known to contain growth-promoting and stimulating properties and is a satisfactory media for the tissue culture of mammalian cells. *In vitro* this may be the result of a ready supply of preformed nucleo-proteins(4-6).

With the possibility in mind that liver nucleo-protein may have been modified, liver sections from 8 chicks of groups (a), (b), and (d) were analyzed for their ribonucleoprotein content. The standard technic of toluidine blue staining before and after ribonuclease digestion of comparable sections was used(7). No differences in the liver ribonucleoprotein content were found by this technic in the livers from the 3 groups studied. It would seem unlikely that the liver content of nucleoprotein was effected by the procedures employed in the 3 groups studied.

Summary. Cortisone systemically administered to day-old chicks is a powerful growth

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[*] Supported in part by U.S.P.H.S. through the National Advisory Arthritis and Metabolic Diseases Council.

[†] The Cortisone was kindly furnished by Merck & Co.

inhibitor for the 18-day period of this study. A partial reversal of this growth inhibitory activity of cortisone may be accomplished by the simultaneous administration of beef amniotic fluid.

I wish to thank Ovia Walker, Angela Ramos, and Tom Dillon for their technical assistance.

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Effect of Temperature Variations on Paper Electrophoretic Migration of Serum Lipoproteins.* (22050)

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In a study on filter paper electrophoresis of serum proteins it became quite obvious that temperature changes markedly affected the migration of the serum lipoproteins even though the migration of the main serum proteins was affected to only a negligible extent. In order to determine what variations extremes in laboratory temperature might cause, we have carried out a study on the cholesterol distribution along filter paper strips following electrophoresis at 5°C versus summer room temperature and 35°C. The summer room temperature, though not known exactly, was probably around 26°C to 30°C most of the time.

Methods. A Kelab paper electrophoresis apparatus was used with Whatman #3 filter paper strips (4.6 x 35 cm) and a barbital-sodium acetate buffer of pH 8.6 and ionic strength .05. Three strips of filter paper separated from one another by plastic strips were placed between the glass plates of each of the 2 compartments, thus making a total of 6 strips for each run. To each of the strips at a line 12 cm from the cathode end of the strip a total of 0.05 ml of serum was applied evenly across the strip except for a margin of 2 mm at each edge. The duration of the electrophoresis was 15 hours. A current of 1.2 milli-amps per strip was employed consistently and the potential was approximately 210V. At the end of the electrophoretic procedure all strips were removed and processed as follows: a) 2 strips were oven dried at 100°C for about 45 minutes then stained with bromphenol blue for protein after the method of Köiw *et al.*(1); b) one strip was air dried then stained with Sudan Black-B for lipids; and c) the other 3 strips were kept from drying by suspending them in a 1000 ml glass cylinder containing water at the bottom and covered with an inverted petri dish to reduce evaporation. As soon as the position of the various proteins along the dried strips was determined, which seldom exceeded 90 minutes from the time the strips were removed from the electrophoretic apparatus, the wet strips were cut as indicated below and the sections transferred to 25 ml glass stoppered Erlenmeyer flasks. Nine sections were employed: 1) the albumin area, which also contained the alpha$_1$-globulin; 2) the area between the albumin, alpha$_1$-globulin and the alpha$_2$-globulin; 3) the alpha$_2$-globulin; 4) a narrow strip between the alpha$_2$- and beta-globulins; 5) the beta-globulin; 6) a strip between the beta-globulin and 5 mm from the starting point; 7) the area from 5 mm of the starting point to 5 mm on the other side of the starting point

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TABLE I. Effect of Temperature Variations on the Electrophoresis of Serum Lipoproteins.

	Avg % of total cholesterol/section with stand. dev.								
No. of subjects	Albumin to alpha$_2$	Alpha$_2$	Alpha$_2$ to beta	Beta	Beta to +5 mm	+5 mm to –5 mm	Gamma	Temp., °C.	Total chol.,* mg %
9	19.2±4.2 (12.7-27.4)	10.2±7.4 (2.5-23.3)	4.6±4.6 (0-12.6)	40.6±5.6 (34.3-48.7)	21.6±7.9 (8.2-31.9)	3.1±1.7 (0-6.1)	.6±1.0 (0-3.0)	35†	(175-272)
	18.2±3.6 (13.3-25.1)	3.3±1.4 (.9-5.8)	.2±.6 (0-1.9)	34.6±15.8 (6.5-55.6)	34.2±9.9 (24.5-50.7)	8.1±4.6 (2.3-17.0)	1.1±1.1 (0-4.1)	5	
4	11.1±1.6 (8.8-12.7)	18.4±14.5 (7.9-39.8)	8.3±3.3 (5.3-12.1)	43.2±8.4 (33.5-53.7)	14.4±2.0 (12.1-16.9)	3.7±3.1 (0-7.6)	.9±1.3 (0-2.7)	35†	(331-346)
	12.8±2.4 (9.4-15.1)	4.7±2.9 (2.3-8.8)	1.8±2.2 (0-4.4)	54.5±4.6 (51.5-61.3)	17.3±4.8 (10.4-21.5)	7.1±1.5 (5.7-9.0)	1.7±1.6 (0-3.2)	5	
1	4.9	2.0	6.8	24.3	41.2	19.2	1.3	35	529
	6.4	1.2	3.0	31.4	36.0	21.0	1.0	5	
1	6.5	32.4	3.8	24.4	21.5	9.2	2.1	35	674
	5.8	6.8	6.1	44.0	22.8	12.4	2.0	5	
Approx. width of strip (mm)	45	20	5	22	17	10	10	(blank = 10 mm)	

* Values below 300 mg % are considered normal for total cholesterol; total concentration determined by method of Forbes(2). (Range.)

† 2 in each of these were run at summer room temp. instead of 35°C.

which usually included some of the gamma-globulin; 8) the gamma-globulin; and 9) a portion 10 mm wide towards the end of the strip to serve as a blank. Approximately 17 ml of a 1:1 mixture of ethyl alcohol and ethyl ether were added to each flask, then stoppered, shaken and set aside overnight. On the following day the flasks were immersed to a depth of about 1 inch for 4 hours in a water bath at 63-65°C. Occasionally the solutions boiled slightly at first but sufficient pressure was never developed to blow the stoppers out. Most of the ether had escaped by the end of the period, at which time the flasks were removed from the bath. The solution remaining in each flask was transferred to correspondingly labelled test tubes and the paper and flask rinsed twice with about 3 ml of the alcohol-ether mixture. The tubes were then put in a water bath not above 63°C and the contents evaporated to dryness with the aid of a current of air. Each tube was removed from the bath as soon as dry.

Cholesterol determinations were made on each set of 9 tubes as follows: 2 ml of chloroform and 0.8 ml of acetic anhydride were added to each tube which was then shaken and put in a water bath at 22°C for 10 minutes; 1 drop of concentrated sulfuric acid was added, the tubes shaken, replaced in the water bath and set aside in the dark for 20 minutes for development of the color. The light absorption was read in a Klett-Summerson photoelectric colorimeter using micro cuvettes and light filter #42. A cholesterol standard was run simultaneously. Separate blanks were run for the standard and unknown solutions.

Some experimental results are shown in Table I. Since there was little cholesterol in section 2) the results thereof are combined with section 1). The values in the Table represent the average of 3 strips for each serum. The agreement between strips was quite good and the average of the sum of the 3 strips was usually in close agreement with the total cholesterol. The results on the various subjects with cholesterol below 300 mg per cent have been combined as are also those with values between 300 and 400 mg.

Although the rate of migration of the main serum proteins, as indicated by the bromphenol blue stain, was not significantly affected by the temperature variations employed, the rate of migration of the beta-lipoproteins was quite definitely affected as shown by both the Sudan Black-B staining and the cholesterol distribution. The temperature effect was especially marked in normal sera with normal cholesterol concentration.

The rate of migration of the lipids associated with the albumin, $alpha_1$-globulin area was not apparently affected by the temperature variations. It will be seen from the results shown in Table I that a fair amount of the cholesterol normally associated with the beta-globulin area moved considerably faster at 35°C than at 5°C. The amount of cholesterol remaining around the starting point was usually less at the higher temperature than at 5°C. This was especially evident in serums from normal subjects with normal cholesterol concentrations. The Sudan Black-B staining material in nearly all cases agreed quite well with the cholesterol distribution.

Discussion. The cholesterol distribution which we obtained at 5°C is in reasonably good agreement with that obtained by Boyd (3) using a temperature of 1-12°C. The deterioration of resolution of the $alpha_2$- and beta-globulins which Boyd obtained at higher temperatures was evident in only a few of our cases when electrophoresis was conducted at 35°C, and even in these cases the difference was not marked. The fact that the temperature differences did not apparently affect the rate of migration of the cholesterol-containing lipids found in the albumin, $alpha_1$-globulin area is probably a reflection of their high protein content and great stability as contrasted with the beta-lipoproteins.

Summary. Paper electrophoretic studies showed that the rate of migration of beta-lipoproteins was considerably less at 5°C than at summer room temperature or at 35°C. No effect of temperature was evident in the case of the lipoproteins migrating with the albumin, $alpha_1$-globulin. The rate of movement of the main serum proteins was apparently the same at the different temperatures, but the resolution of the $alpha_2$- and beta-globulins was sometimes less distinct at the higher temperature. The results presented show the importance of using relatively constant temperatures when comparative paper electrophoretic studies are carried out, especially of beta-lipoproteins.

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Effect of Hg^{203}-Labeled Mercaptomerin on Renal Sulfhydryl Concentration in Normal Rabbits.* (22051)

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When labeled organic mercurials are injected intravenously into normal animals, the radioactivity is localized in the kidney(1,2). The purposes of the present study were to measure the concentration of mercury in the kidneys of normal rabbits following the administration of large doses of the tagged mercurial, and to determine the effect of this agent on the renal concentration of sulfhydryl compounds.

Material and methods. Mercaptomerin sodium‡ was labeled with Hg^{203} to contain 40 μc of Hg^{203} per mg of mercury; each ml of the stock solution contained 40 mg of mercury in 140 mg of mercaptomerin sodium. Fourteen young rabbits were used. Each of 12 animals was anesthetized with ether and a unilateral nephrectomy was performed. The desired

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† Established Investigator of the American Heart Assn.

Hg^{203} supplied by Oak Ridge National Lab., Oak Ridge, Tenn., on allocation from U. S. Atomic Energy Commission.

‡ Hg^{203}-labeled mercaptomerin sodium was synthesized by the Wyeth Institute of Applied Biochemistry.

TABLE I. Distribution of Hg^{203}-Labeled Mercaptomerin Sodium in Rabbit Kidneys and Its Effect on the Sulfhydryl Content.

I.V. dose of mercaptomerin (mg Hg/kg)	Conc. of mercury (mg/g dry wt tissue)		Serum Hg conc. (μg/ml)	Conc. of sulfhydryl (mg/g dry wt tissue)	
	Cortex	Medulla		Control kidney cortex	Test kidney cortex
17.9	1.20	.20	78.5	12.9	8.6
16.7	1.90	.70	46.0	7.8	5.4
19.6	1.90	.70	42.9	10.7	8.4
20.9	2.40	1.40	85.7	12.5	5.7
22.0	2.60	.80	—	10.8	6.4
32.5	2.60	.30	—	8.6	7.8
14.8	2.70	.50	—	9.2	7.5
26.9	2.70	.90	74.0	9.3	5.8
20.9	2.80	—	—	10.1	5.3
14.3	3.10	1.50	—	11.0	7.1
30.2	3.10	.90	96.5	8.4	6.4
16.8	3.40	.60	32.5	9.6	6.7
0	0	0	0	11.3	12.2
0	0	0	0	8.3	12.2
Mean values	2.53	.77		10.08	6.76

dose of tagged mercaptomerin (ranging from 14 to 30 mg of mercury per kilo of body weight) was then immediately injected into a marginal vein of the ear, and ether anesthesia was continued for another hour. Exactly one hr after injection of the mercurial, the animal was killed and the remaining kidney was removed. Two animals were used as controls and were treated in the same manner as the test animals, except that the injection of the mercurial was omitted. The cortex and medulla were separated by gross dissection. Samples from each, weighing approximately 1 g, were weighed and homogenized to a 5% suspension (weight to volume) in a phosphate buffer at pH 7.5 in the Elvejhem-Potter glass homogenizer. Three 1-ml aliquots of each of the 5% homogenates were analyzed for sulfhydryl content by the method of Anson (3). The SH concentrations found in the tissues have been expressed in terms of the 1-cysteine equivalent per g dry weight of tissue. Another 1-ml aliquot of each of the 5% homogenates was placed in a test tube of known weight and was assayed for radioactivity content. The test tubes were then dried at 110°C until constant weights were reached. The precipitates obtained after the addition of trichloracetic acid were decanted free of supernatant fluid, and were also assayed for radioactivity content.

Results. Table I. The concentration of mercury in the cortex varied between 1.2 and 3.4 mg/g of tissue (dry weight), with a mean value of 2.53 mg/g. These figures are considerably higher than the values found in the medulla, which ranged from 0.2 to 1.5 mg/g, with a mean of 0.77 mg/g. From 90 to 98% of the radioactivity found in the homogenates was recovered in the trichloracetic acid precipitate; the mean value was 94%.

The sulfhydryl concentrations in the renal cortices removed before injection of the mercurial ranged between 7.8 and 12.9 mg, expressed as 1-cysteine/g dry weight of tissue, with a mean value of 10.08 mg/g. In all 12 instances in which labeled mercaptomerin was administered intravenously, the sulfhydryl concentration in the test kidney (5.3-8.6 mg/g, with a mean value of 6.76 mg/g) was lower than that in the control kidney. In the 2 control animals not given mercaptomerin, however, the SH values increased during the period of anesthesia. The results of the SH determinations on the renal medulla were not reliable, due to technical difficulties, and have not been reported.

Comment. That Hg^{203} has a predilection for the renal cortex is evident from the fact that cortical concentrations of mercury were, on the average, 3.3 times greater than those in the medulla. This distribution would be expected if the mercurial had localized in the proximal tubules, as others have found in histochemical studies(4). Since most of the radioactivity was recovered in the trichloracetic acid precipitate, it can be inferred that the mercury was bound to renal tissue proteins.

The results of the present study show that the intravenous administration of organically bound mercury is associated with a decrease in the renal cortical concentration of sulfhydryl radicals.

Summary. Hg^{203}-labeled mercaptomerin sodium, in a dosage of 14 to 30 mg of mercury/kg of body weight, was injected intravenously into normal rabbits immediately following a unilateral nephrectomy, and the remaining kidney was removed one hour later. The mean cortical and medullary concentrations of mercury, were, respectively, 2.53 and 0.77 mg/g of dry weight of tissue. The mean sulfhydryl concentration in the control cortices was 10.08 mg/g of dry tissue, expressed as 1-cysteine; the mean value obtained one hour after administration of the tagged organic mercurial agent was 6.76 mg/g. The SH concentration of the cortex decreased in all 12 test animals following the injection of labeled mercaptomerin, and increased in the 2 uninjected control animals.

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Effect of Thyroxine and Related Compounds on Heparin-Activated Fatty Acid Liberating Enzyme.* (22052)

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Plasma from a human given intravenous sodium heparin contains an enzyme capable of hydrolyzing the triglycerides of egg lipoproteins and human S_f 20-400 lipoproteins, the latter both *in vitro* and *in vivo,* releasing fatty acids and increasing the S_f 4-20 lipoprotein level of the plasma(1-3). The lipoprotein transformations involve primarily the lipid components since the protein components of the S_f 4-20 and S_f 20-400 lipoproteins of normal humans seem to be of quantitatively identical amino acid composition(4). No definite evidence exists that the heparin-activated enzyme normally mediates lipoprotein metabolism, though Jeffries(5) has demonstrated the presence of a very similar, if not identical, enzyme in the rat following olive oil administration, and Korn(6) has obtained from rat heart an enzyme catalyzing the hydrolysis of the neutral fat of chylomicra. Though very much less susceptible to hydrolysis by the heparin enzyme, human S_f 4-20 lipoproteins can be significantly lowered in concentration by the administration of desiccated thyroid(7,8). For these reasons, and particularly if the heparin-activated enzyme is physiologically important (requiring for its activity no elements not normally present in the body), it seemed of interest to examine the effect of thyroxine and related compounds on the triglyceride-splitting enzyme.

Materials and methods. Enzyme (preheparin serum or post-heparin plasma) and human or egg lipoprotein substrate were prepared as described previously(1). L-thyroxine (THRX, obtained from Smith, Kline, French Laboratories and from Sigma Chemical Co.), L-triiodothyronine (TRIT, obtained from Smith, Kline, French), D-diiodothyronine (DIIT, obtained from Wyeth Laboratories, Inc.), and related compounds were dissolved in a minimum volume of 0.1 N sodium hydroxide and added to serum or plasma, the pH being adjusted to 8.0 with 0.1 N hydrochloric acid and the ionic strength made identical in all cases with 0.1 N sodium chloride. The calci-

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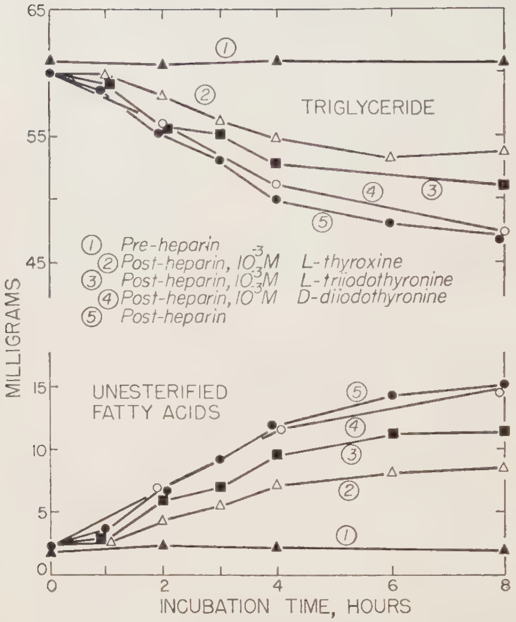

FIG. 1. Effect of thyroxine and related compounds on fatty acid liberation from egg lipoprotein triglycerides by post-heparin plasma.

um and magnesium experiments were carried out by adding the THRX-enzyme solution to substrate-cation solution and by adding THRX-enzyme-cation solution to substrate. In all experiments, after incubation of enzyme and inhibitor for 30 minutes at room temperature, the lipoprotein substrate was added and the contents incubated at 37.5°C. The pH remained constant in all experiments except for the 0.2-0.4 unit lowering brought about by the liberated fatty acid. At the end of the incubation, the contents were extracted with methanol-ethyl ether, the lipids separated chromatographically on silicic acid, and the fractions analyzed by infrared spectrophotometry(1).

Results. Effect of THRX and related compounds on fatty acid release. The data of Fig. 1 and of Table I indicate that THRX and TRIT inhibit the heparin-activated enzyme, the former compound being about 3 times more effective than the latter at equimolar concentrations. DIIT, L-tyrosine, and sodium iodide do not inhibit. Calcium or magnesium ion (3×10^{-3}M) or heparin (9×10^{-4} M, a concentration at least 400-fold that present in plasma after administration of 100 mg heparin) do not reverse the inhibition. In titrations carried out by the method of Alper(9), 3.9 ml 0.01 N sodium hydroxide were required to titrate the butyric acid liberated from tributyrin by post-heparin plasma, 3.7 ml that liberated by pre-heparin serum, 2.6 ml that liberated by post-heparin plasma 1×10^{-3} M in THRX, and 3.3 ml that liberated by post-heparin plasma 3×10^{-4} M in THRX. All the titrations were carried out in triplicate, with an uncertainty of 5-10%.

Effect of oral administration of THRX on fatty acid release and lipoprotein transformations. Before THRX administration, and after 10 mg intravenous heparin each day, the plasma of a normal individual at intervals of 2 days liberated 14.1, 13.4, and 13.9 mg fatty acid from 65 mg egg lipoprotein triglyceride prepared from different eggs. After daily oral administration of THRX (Smith, Kline, French tablets) for 8 days, 0.6 mg the first 2 and 1.0 mg thereafter, and following injection of 10 mg heparin at 2 times, the second 10 hours after the first, the plasma liberated 10.5 and 10.8 mg fatty acid, respectively from 66 mg lipoprotein triglyceride. During THRX consumption, the protein-bound iodine value rose from 5.6 to 14.9 μg/100 ml, corresponding at most to a THRX level of 2×10^{-6} M. At the conclusion of the experiment, THRX added to the plasma to a final concentration of 1×10^{-4} M was as effective in inhibiting fatty acid release from egg lipoprotein substrate as THRX added to a final concentration of 1×10^{-3} M had been before oral THRX consumption, 12.0 (10^{-3} THRX) and 12.5% (10^{-4} THRX) of the substrate triglyceride being hydrolyzed by THRX-containing, post-heparin plasma and 19.8% by the non-THRX-containing, pre-THRX administration post-heparin plasma.

The fasting standard S_f 0-20, 20-100, and 100-400 lipoprotein levels of the subject before THRX administration were 263, 54, and 21 mg%, respectively; after 10 mg intravenous heparin (Lederle) and withdrawal of the blood 10 minutes later, these values had become 288, 7, and 0 mg%. At the conclusion of THRX administration, these levels had become 129, 51, and 19 mg%, respectively; 10 mg intravenous heparin changed these levels to 144,

TABLE I. Liberation of Fatty Acid from Lipoprotein Triglycerides after Incubation with Pre-heparin Serum or Post-heparin Plasma and Various Compounds at 37.5°C for 4 Hours.

Incubation constituents	mg fatty acid increase*	$100 \times \frac{\text{mg fatty acid increase}}{\text{mg triglyceride in substrate}}$
(1) 2 ml S_f 20-400 lipoprotein (20-400 lp)	14.0	23.5
(2) 20-400 lp, 0.02 mg s. h.	.4	.6
(3) *Idem*	.5	.7
(1) " , 1×10^{-3} M L-thyroxine	7.6	12.7
(1) " , 3×10^{-4} M *Idem*	10.0	16.7
(1) " , 1×10^{-4} M "	12.0	20.1
(1) " , 1×10^{-3} M " , 9×10^{-4} M heparin	7.8	13.1
(2) " , 1×10^{-3} M "	.3	.5
(1) " , 1×10^{-3} M L-triiodothyronine	10.8	18.1
(1) " , 3×10^{-4} M *Idem*	12.3	20.6
(1) " , 1×10^{-3} M D-diiodothyronine	13.6	22.8
(1) " , 1×10^{-3} M L-tyrosine	13.7	22.9
(1) " , 1×10^{-3} M Sodium iodide	13.7	22.9
(2) " , 1×10^{-3} M L-triiodothyronine	.3	.5
(1) 2 ml egg lipoprotein (elp)	15.2	16.1
(1) elp, 1×10^{-3} M L-triiodothyronine	12.2	12.9
(1) " , 1×10^{-3} M L-thyroxine (THRX)	8.9	9.5
(2) " , 1×10^{-3} M THRX	.5	.6
(1) " , *Idem* , 3×10^{-3} M $CaCl_2$†	9.6	10.2
(1) " , " , 3×10^{-3} M $CaCl_2$‡	9.0	9.5
(1) " , " , 3×10^{-3} M $MgCl_2$†	9.3	9.8
(1) " , " , 3×10^{-3} M $MgCl_2$‡	9.3	9.8
(1) " , 3×10^{-3} M $CaCl_2$	16.0	16.9
(1) " , 3×10^{-3} M $MgCl_2$	15.5	16.4
(2) " , 3×10^{-3} M $CaCl_2$	.7	.8
(1) " , 1×10^{-3} M disodium versenate,§ pH 8.0	14.0	14.7
(1) " , 1×10^{-3} M sodium citrate, pH 8.0	13.9	14.6
(1) " , 1×10^{-2} M *Idem*	9.8	10.4

* The fatty acids produced came from the triglycerides, with no significant phospholipid or cholesterol ester hydrolysis to fatty acids. The values given represent averages of at least 4 determinations, with a reproducibility of 5-10%.

† Cation added to enzyme before enzyme addition to substrate.

‡ " " " substrate " " " " " .

§ Disodium versenate = disodium salt of ethylene diamine tetracetic acid dihydrate.

(1) = 3 ml post-heparin plasma; (2) = 3 ml pre-heparin serum, s. h. = sodium heparin and present in all incubations with (2); (3) = 3 ml pre-heparin plasma, obtained by drawing blood into a heparinized syringe.

21, and 6 mg%. All the experiments on fatty acid release and lipoprotein transformation ability were repeated with two volunteers given 3 grains of desiccated thyroid (Armour) daily for 3 days (until complaint of unpleasantness); at most, a 5-10% inhibition was observed though the S_f 0-20 lipoprotein level had been reduced 15%.

Discussion. The approximate 3-fold superiority of THRX to TRIT in inhibiting fatty acid release is in conflict with the usual relative effectiveness of these compounds in biological systems, the latter being at least as effective and quite often 2 to 10 times more effective in mediating reactions(10). In view of the antagonism between the iodinated amino acids and magnesium (THRX binding the cation more strongly than does TRIT (11)), and in view of the role of calcium and magnesium cations, albumin(12-14), or lipoproteins(15) as fatty acid acceptors and facilitators of triglyceride hydrolysis, the experiments with calcium, magnesium and THRX were performed. Failure after cation addition to reverse significantly the thyroxine-mediated inhibition argues against a calcium or magnesium ion-amino acid complex as the cause of the inhibition. In addition, the data in Table I indicate that the potential cation-complexing agents, citrate and versene (10^{-3} M) are much less effective than the iodinated amino acids (10^{-3} M) as inhibitors of fatty acid release. Since heparin addition does not reverse inhibition, the amino acids probably do not function by heparin removal.

Many workers(16-21) have presented data that there appears to be an α-globulin component in human serum with a binding mechanism so specific for THRX that TRIT fits less well and DIIT is excluded. If the heparin-activated enzyme is of such nature that THRX is bound to it more strongly than TRIT, or if the concentration of the latter available for inhibition is reduced by combination with non-enzymic plasma proteins, then the latter amino acid could be less inhibitory than the former. Our kinetic studies are not of sufficient accuracy to indicate amino acid-enzyme or amino acid-substrate binding. Inhibition of both tributyrin and lipoprotein triglyceride hydrolysis may be a reflection of non-specific esteratic enzyme site binding. The relatively high concentrations of THRX needed for inhibition in our experiments and the failure to observe an increase in S_f 20-400 lipoprotein levels accompanying the decrease in S_f 4-20 levels in patients given desiccated thyroid(7)—the increase to be expected if the thyroid is a natural antagonist of the heparin enzyme and if the enzyme itself is physiological—make it premature at present to associate the observed thyroxine effects with physiological thyroid function. Cairns and Constantinides (22), using amounts of THRX far greater (0.2 mg/rat/day for 8 days) than those used by us, found that THRX inhibited the "lipemia-clearing activity" induced by heparin injection, and that thyroidectomy alone failed to accelerate the activity, concluding that the THRX effect was not physiological.

Summary. 1. L-thyroxine and L-triiodothyronine inhibit the heparin-catalyzed release of fatty acid from lipoprotein triglycerides, the former inhibiting significantly at 3×10^{-4} M and being about 3-fold more effective than the latter at equimolar concentrations. 2. The inhibition does not result from removal of calcium or magnesium ions or heparin.

We have profited from discussions with Drs. J. W. Gofman and T. R. E. Pilkington. We wish to thank Smith, Kline, French Laboratories (L-thyroxine and L-triiodothyronine), Lederle Laboratories (sodium heparin), and Wyeth Laboratories (D-diiodothyronine) for their generous gifts of pharmaceuticals.

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Morphological Changes in Thymus of Rats Following Whole-Body Exposure to Massive Doses of Radiation.* (22053)

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During the course of studies on the biochemical effects of ionizing radiation we observed that more thymic tissue was present in young rats after exposure to over 10,000 r of X-radiation than in comparable animals which had received lesser dosages. Following this observation, a study was made of the histological changes in thymus glands of rats given massive doses of radiation and attempts were made to elucidate the nature of the response. The present communication contains the results of experiments which have clearly shown that massive, whole-body irradiation induces only minimal cellular destruction in the thymus, in contrast to the more pronounced cytological changes that follow exposure to smaller dosages. In addition, they provide evidence to indicate that exposure of the head region plays an important role in the pathogenesis of the paradoxical cellular response in the thymus.

Materials and methods. For the studies the following general plan was used. Randomly selected groups of 5 to 10 rats were exposed over the entire body to X-ray or gamma radiation in doses between 400 and 30,000 r. The animals of these and all subsequent groups were sacrificed 24 hours after the completion of the exposures. The thymus, spleen, and adrenal glands were weighed, fixed in 10% formalin or Bouin's solution and, together with a number of pituitary glands and brains, were studied in detail by histological means. In a study of the localization of the tissues that govern the thymocytic response, groups of rats were exposed locally over the mediastinal region to graded doses of radiation and other groups of animals were given radiation over the body with the head shielded. To investigate the temporal aspects of this biological response, groups of rats were first given a small dose of total-body radiation and at varied times thereafter were re-exposed to massive doses. Twenty-five normal rats, together with groups of anesthetized and non-anesthetized irradiated animals were sacrificed for control purposes. Young female rats of the Sprague-Dawley strain were used. All were approximately 3 months of age and had weights of 200 ± 20 g. They were maintained throughout the experimental period in air-conditioned quarters with free access to Rockland rat pellets and water. X-radiation was administered at a rate of 320 ± 20 r/min. by a Keleket Therapy Unit operated under the following conditions: 250 KV, 15 ma, and filtration of 0.25 mm of Cu and 1.0 mm Al. Gamma radiation was administered from a Co^{60} source at a rate of 1,000 ± 50 r/min. To obtain localized exposure of the thymic area, the animals were anesthetized (sodium pentobarbital, 35 mg/kg) and irradiated within a 0.25-in. thick lead cylinder which had a single 1.0 by 0.5 in. aperture over the mediastinal region. To restrict gamma radiation to the body, the head was shielded with 0.62 in. of lead and the rays were collimated through concrete. There was less than 25% scatter of radiation to the head, with the highest dosage. The radiation fields and dose rates were calculated in air with phantoms in position. Measurements were checked with a high range Co^{60} rate meter, a 2,500 r Victoreen thimble chamber, $FeSO_4$, and one- and 2-phase chemical dosimeters.

Results. Single-dose, massive, whole-body irradiation. The mean weights of the thymuses of groups of rats at 24 hours after total-body exposure to doses of X-radiation from

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* This study was conducted at the Radiobiological Laboratory of The University of Texas and the USAF, Austin, Texas and the USAF Radiation Laboratory, University of Chicago. It was supported (in part) with funds provided under Contracts AF 18(600)-165 and AF-33(038)27353, and the School of Aviation Medicine.

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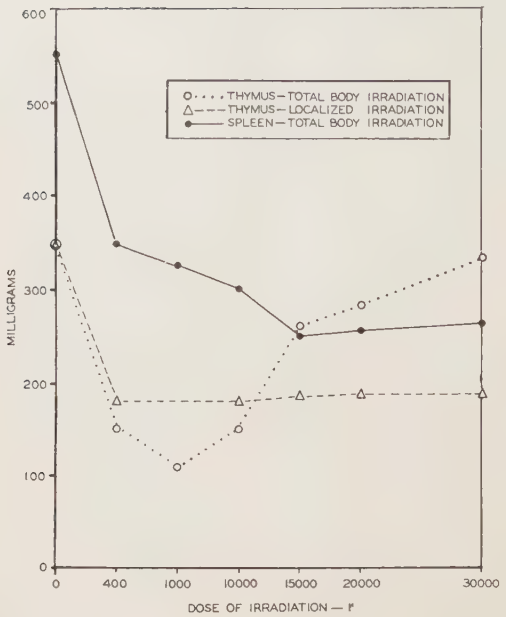

FIG. 1. Thymic and splenic weights in rats 24 hr after X-radiation of entire body and localized to mediastinal area. Each point is mean wt of organs from 5 to 10 animals.

400 to 30,000 r are shown graphically in Fig. 1. Following exposure to 400 r the thymus decreased within a 24-hour period to less than one-half its normal weight (mean weights, 361 to 149 mg). The weight loss was slightly greater after exposure to 1000 or 10,000 r. However, the administration of doses greater than 10,000 r was regularly followed by significantly less weight loss, with 30,000 r inducing only a 35 mg alteration in the mass of the thymus. A very close correlation existed between the weights of the thymuses of rats exposed to gamma radiation and those of animals administered the same quantity of X-radiation (Fig. 2). By contrast, the weight loss that was incurred in the spleens of these animals was regularly marked after all dosages, while the weights of the adrenal glands were not altered significantly from the normal. The weights of the thymus in anesthetized and non-anesthetized control groups were essentially identical.

In histological sections, the thymic tissues of rats exposed over the entire body to massive doses of X- or gamma radiation showed a conspicuous preservation of lymphocytes as compared with tissues of animals exposed to lesser dosages (Fig. 3). The preservation of cells was more evident in the cortex than in the medullary regions. In general, a very close correlation existed between the degree of cellular destruction, the width of the cortical layer, and the weight of the gland. The nuclei of many of the lymphocytes were pyknotic; nevertheless, the nuclear membranes remained intact and the chromatin material was particulate, being neither fused nor fragmented. Although karyorrhexis and phagocytosis were prominent features in the spleens of the same animals and in animals exposed to smaller dosages, they were inconspicuous or absent in the thymuses of rats exposed over the entire body to massive doses (Fig. 4). The adrenal and pituitary glands, as well as the central nervous system, with the exception of the granule cells of the cerebellum, showed no consistent morphological alteration attributable to radiation, the findings differing from those previously observed in monkeys(1).

Studies of the mechanism. When the mediastinal region of rats was exposed to localized X-radiation, the degree of cytological change

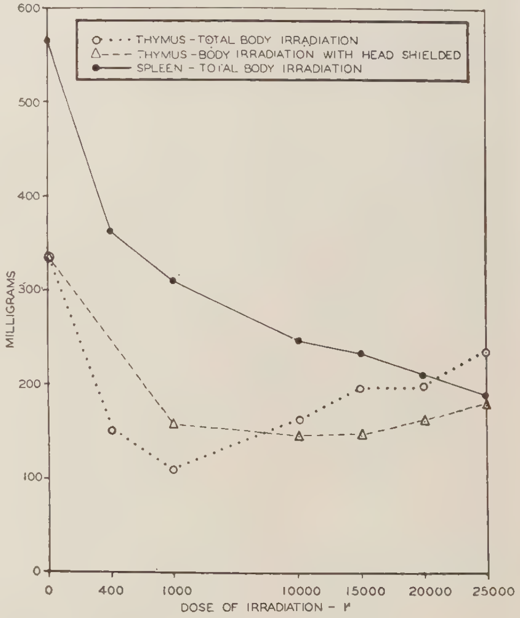

FIG. 2. Thymic and splenic weights in rats 24 hr after gamma radiation of entire body and localized to the body with the head shielded. Each point is mean wt of organs from 5 to 10 animals.

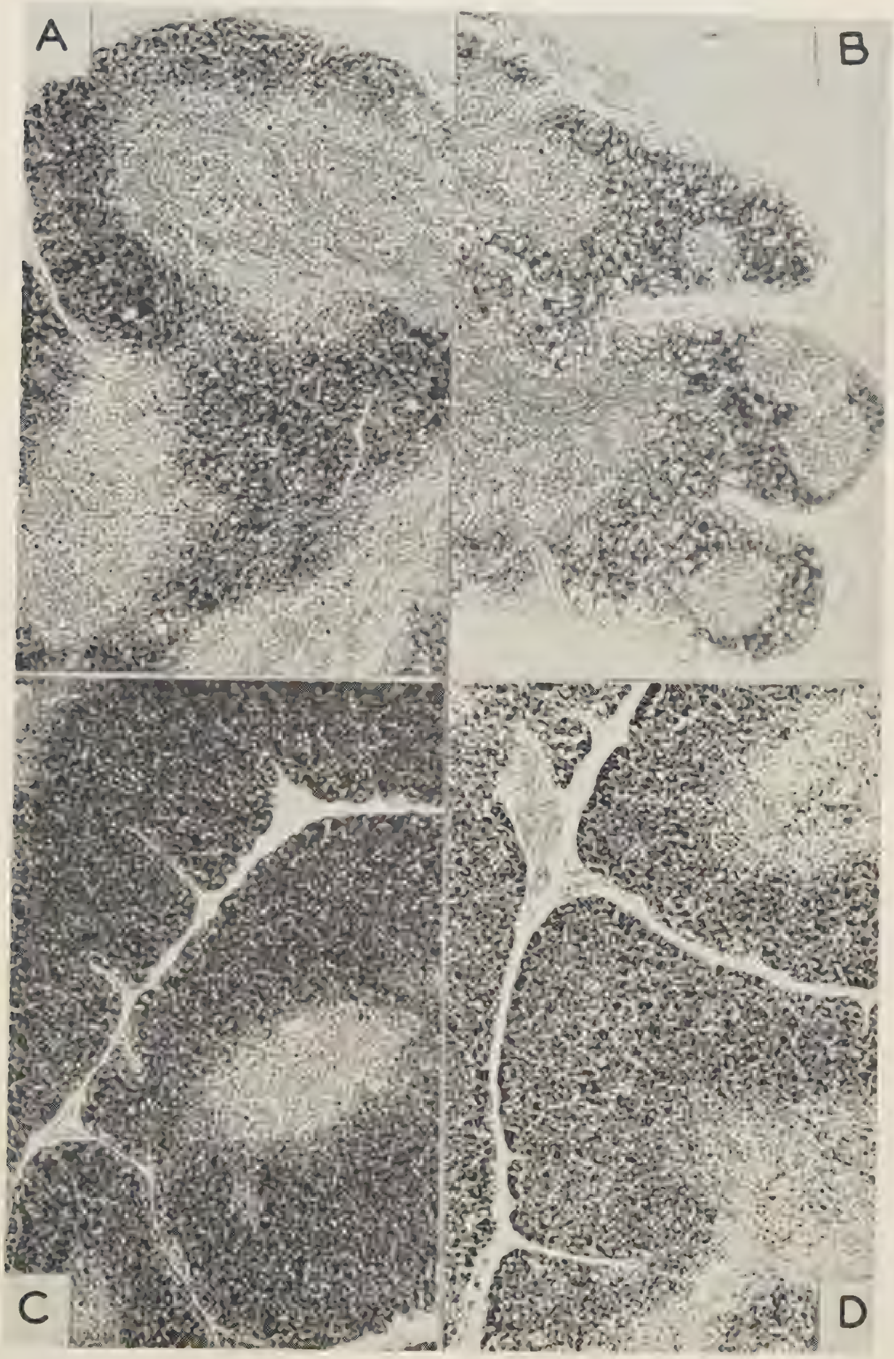

FIG. 3. Thymus of rats 24 hr after total-body exposure to X-radiation. Hematoxylin and eosin stain. × 50. A. (400 r exposure.) Cortex is narrowed, markedly depleted of lymphocytes, and has an avg thickness of .16 mm (normal = .52 mm). B. (10,000 r exposure.) Cortex is almost devoid of lymphocytes and has an avg thickness of .14 mm. C. (20,000 r exposure.) Cortex is of approximately normal thickness and is densely populated with lymphocytes. It measures on the avg .49 mm across. D. (30,000 r exposure.) Cortex is highly cellular and measures approximately .50 mm in width.

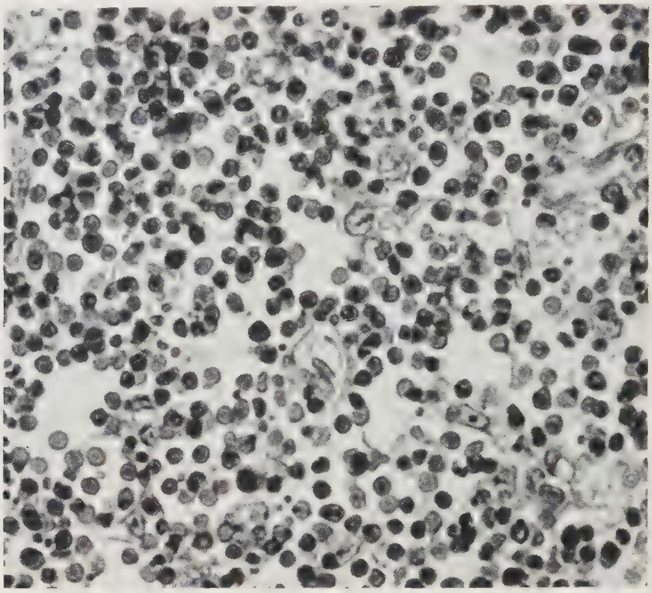

FIG. 4. (Higher magnification of Fig. 3D.) Lymphocytes in cortex are approximately normal in concentration although many are pyknotic. Karyorrhexis and phagocytosis are inconspicuous. Hematoxylin and eosin stain. × 800.

and weight loss in the thymus was essentially constant after all dosages above 400 r (Fig. 1). This finding provides evidence that the cytological response of the thymic lymphocytes to massive, whole-body irradiation is modified by the exposure of tissues outside the mediastinal area.

When the body alone was exposed to high intensity radiation with the head shielded, the degree of cytological destruction and weight loss in the thymus was significantly increased as compared with that induced by total-body exposure to the same dose of radiation (Fig. 2). This finding suggests that, during massive whole-body irradiation, exposure of the head plays an important role in the pathogenesis of the unusual cellular response in the thymus.

In groups of rats exposed to 400 r of whole-body radiation and then re-exposed within one hour to 19,600 r, the weight loss and cytological change in the thymus approximated that in animals exposed to a single, uninterrupted dose of 20,000 r. When the interval between exposures was lengthened to 6 hours, the weight loss in the thymus was markedly increased and equalled or exceeded that in animals exposed to a single dose of 400 r (Fig. 5).

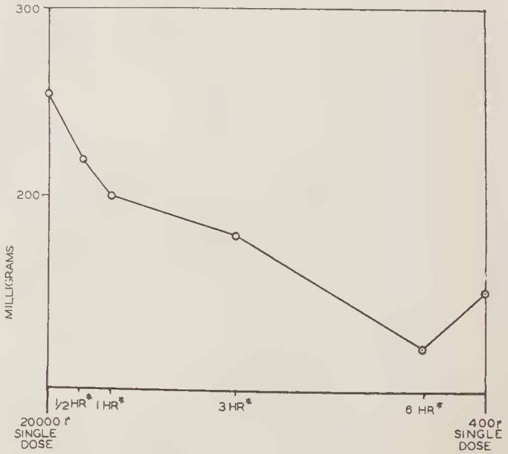

FIG. 5. Thymic weights in rats 24 hr after divided doses of 20,000 r of total-body X-radiation. Each point is the mean wt of organs from 5 to 10 animals. Asterisk indicates time lapse between administration of an initial dose of 400 r and a subsequent dose of 19,600 r.

Summary. The weight loss and degree of cytological alteration in the thymuses of rats was less after whole-body exposure to quantities of X- or γ radiation above 10,000 r than after exposure to smaller dosages. Administration of 10,000 to 30,000 r of localized radiation to the thymic region, or to the body with the head shielded, was regularly followed by significantly greater cellular destruction and weight loss in the thymus than occurred after whole-body exposure to the same dose of radiation. Whole-body re-exposure to a massive dose of radiation within one hour after an initial total-body exposure to a small dose was regularly followed by less weight loss and cellular destruction in the thymus than occurred after total-body exposure to the small dose alone. The findings show that exposure of the head during massive whole-body irradiation markedly influences the cellular response in the thymus.

The authors wish to acknowledge the valuable assistance of Dr. Kenneth P. DuBois in these studies.

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Effects of Oxidized Fatty Acids on Ascites Tumor Metabolism. (22054)

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The unsaturated fatty acids of cells and mitochondria have been shown to be oxidized by ultraviolet light(1,2) and by aerobic incubation(3). The oxidation products of these fatty acids quite probably alter cellular activities as indicated by the findings that the unsaturated fatty acid esters, methyl linolenate and methyl linoleate, inhibit the activity of certain oxidative enzymes in homogenates and mitochondria(2,3); depolymerize deoxyribonucleic acid (DNA)(4); and inhibit cell division(5,6). The relation of oxidized fatty acids to enzyme action is further examined in the present study of the action of oxidation products of linolenate and linoleate on the respiration and glycolysis of Ehrlich ascites tumor cells.

Methods and materials. Ehrlich ascites tumor growing in A-strain mice was taken from the abdominal cavity 7 days after injection. Blood-free tumor cell suspensions were prepared by centrifuging the ascitic fluid first for 10 minutes at 100 x g. The cells were then resuspended in Ringer solution and centrifuged at 2 x g for 15 minutes. The supernate which contains erythrocytes and platelets was discarded and the tumor cells resuspended in Ringer solution. This procedure was repeated 3 times. For testing the effects of oxidized fatty acids, aqueous extracts containing the oxidation products were made from pure fatty acid esters (Hormel) oxidized by irradiation with ultraviolet light. Samples of 0.5 g of the esters were irradiated for 90 minutes in dishes 3 cm in diameter placed at a distance of 30 cm from a Hanovia Utility Model mercury arc rated at an intensity of more than 250 microwatts/cm^2 at 50.8 cm distance for wavelengths of 3130 Å and shorter. 0.5 g of the irradiated fatty acid ester was dissolved in 10 ml of petroleum ether and extracted against an equal volume of distilled water with shaking for 20 minutes. After separation of the petroleum ether the aqueous phase was centrifuged under fresh petroleum ether to remove any suspended droplets of ether. The aqueous phase was then placed under vacuum for 20 minutes to remove traces of ether. Two volumes of the aqueous phase were diluted with one volume of 3 x isotonic Ringer solution and varying amounts of this mixture were added to Warburg flasks to give the desired amount of oxidized material as determined by the thiobarbituric acid (TBA) color reaction(7). The weight of oxidized ester cannot be calculated but is of the order of micrograms. The respiratory and glycolytic measurements were made by standard Warburg technics. Lactic acid formed aerobically was measured by the method of Barker and Summerson(8). Nitrogen was determined by digestion and nesslerization. Analysis of the unsaturated fatty acids of tumor cells was made on lipid of several grams of packed cells extracted with a 3:1 alcohol-ether mixture for 12 hours in a Soxhlet type extraction appara-

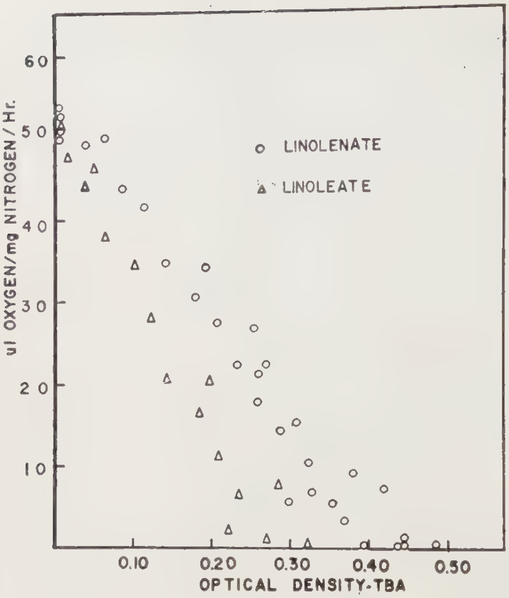

FIG. 1. Effects of aqueous extracts of oxidized methyl linolenate and methyl linoleate on respiratory activity of ascites tumor cells. Flasks contents: 1.0 ml of cell suspension containing about 5×10^7 cells; 2.0 ml of Krebs Ringer phosphate solution. Aqueous extracts of linolenate and linoleate were added with the Ringer solution. TBA values represent total amount of added oxidized esters. Measurements were taken for one hr.

tus. The percentage of each unsaturated fatty acid was measured after alkaline conjugation by the spectrophotometric micromethod of Herb and Riemenschneider(9). The iodine number was determined by the method of Benham and Klee(10).

Results. Respiratory inhibition. Washed tumor cell preparations exhibited a constant rate of oxygen consumption for an hour or longer followed by a gradual decrease in respiration over several hours. $Qo_2(N)$ values during the first hour were between 50.0 and 54.5 as compared with a value of 58 reported by Kun *et al.*(11). The addition of small quantities of aqueous extracts of oxidized methyl linolenate and linoleate caused a decrease in respiratory activity which was a linear function of the concentration of extract (Fig. 1). The inhibition reached a maximum in the first intervals of the respiratory measurements. Inhibition by oxidized methyl linolenate was usually complete at concentrations giving TBA optical densities greater than 0.400. Extracts of oxidized linoleate inhibited oxygen consumption at lower TBA values than oxidized linolenate. However, since the TBA reaction is 30-80 fold more sensitive to oxidized linolenate as compared to oxidized linoleate(7), oxidized linoleate is actually a less potent inhibitor than oxidized linolenate.

Inhibition of glycolysis. Warburg and Hiepler(12) and Kun *et al.*(11) showed that ascites cells exhibited high rates of lactic acid formation in the presence of glucose under both aerobic and anaerobic conditions. The addition of aqueous extracts of oxidized linolenate to aerobic and anaerobic glycolyzing cells decreased the rate of lactic acid formation. The degree of inhibition was proportional to the amount of extract added in excess of a certain threshold concentration which was about the same for both aerobic and anaerobic glycolysis (Fig. 2). The inhibition appears to be slightly greater for aerobic glycolysis at higher concentrations of extract. It is to be noted that the respiration was inhibited completely at the threshold concentration for the inhibition of glycolysis (Fig. 1). The effect of oxidized methyl linoleate extracts followed the same general pattern observed for oxidized methyl linolenate (Fig. 3). The TBA values of oxidized linoleate extracts for any given degree of inhibition are lower than in the case of oxidized linolenate, but because of the difference in sensitivity of the TBA reaction to the 2 esters, higher concentrations of oxidized linoleate are required.

Fatty acid oxidation in the cell. The inhibition of respiration and glycolysis by oxidized unsaturated fatty acid extracts raises the problem of similar effects resulting from the oxidation of unsaturated fatty acids normally present in cells. Accordingly, attempts were made to oxidize the fatty acids within the Ehrlich ascites tumor cells under conditions which bring about fatty acid oxidation in other tissues. Also, analyses of the unsaturated fatty acid content of the cells were carried out. Tumor homogenates in Na-K phosphate buffer at pH 6.7 were shaken in air in a Dubnoff metabolic shaker, and after one hour the extent of lipid oxidation was measured by the TBA reaction. Different concentrations of homogenate were run over a

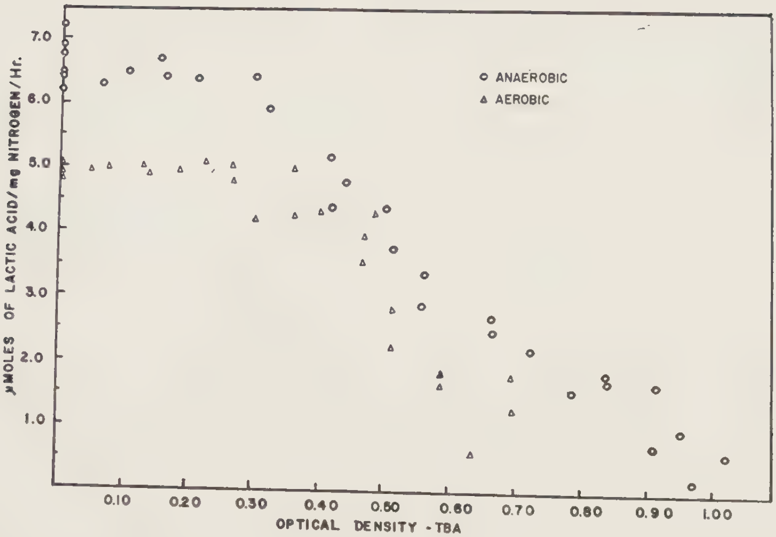

FIG. 2. Effects of aqueous extracts of oxidized methyl linolenate on anaerobic and aerobic glycolysis of ascites tumor cells. In anaerobic measurements each flask contained 1 ml of cell suspension containing approximately 2.5×10^7 cells, 2.0 ml of Krebs Ringer bicarbonate solution, and 0.2 ml of glucose solution (16.6 micromoles). Flasks were flushed with 95% N_2-5% CO_2 mixture, and lactic acid was measured by CO_2 evolution. In aerobic measurements Krebs Ringer phosphate solution was used; the flasks were flushed with 95% O_2-5% CO_2; and lactic acid was measured by chemical determination. TBA values represent total amount of added oxidized methyl linolenate. Measurements were taken for one hr.

range of 25 mg/ml to 250 mg/ml both with and without the addition of 0.3 mg/ml of ascorbic acid as a catalyst for lipid oxidation (13). Tumor homogenates were also irradiated with ultraviolet light and the oxidation products of fatty acids determined by the TBA reaction. 5 ml of homogenate were exposed for intervals up to 2 hours slowly rotating in spherical quartz flasks 6.4 cm in diameter placed 26 cm from the mercury arc used to oxidize the fatty acid esters. The ultraviolet dose was greatly in excess of that required to oxidize lipids of mitochondria(2). In no case could significant fatty acid oxidation be detected. In contrast to tumor tissue, homogenates of mouse brain, liver, and kidney produced high values on incubation under identical conditions. Analyses of lipids of ascites cells are given in Table I. The figures are mean values of 3 replications on each of 2 lots of washed tumor cells, each consisting of the pooled tumor cells from several mice. The proportions of unsaturated fatty acids differ from rat liver(14) and also from rabbit bone marrow(15) analyzed by the same method. The concentration of linolenic acid is higher in the ascites cells than in rat liver and yet the TBA test is negative in the tumor and positive in liver(16). This indicates an increased antoxidant activity in the tumor.

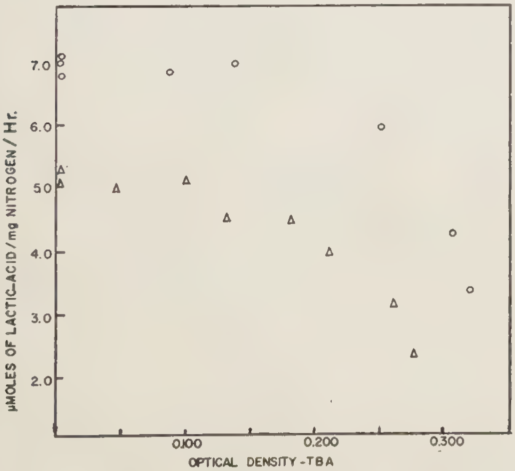

FIG. 3. Effects of aqueous extracts of methyl linoleate on anaerobic and aerobic glycolysis of ascites tumor cells. Experimental conditions as in Fig. 2. ○, anaerobic glycolysis; △, aerobic glycolysis.

Discussion. The inhibition of respiration of intact ascites cells by the oxidation products

TABLE I. Analysis of Unsaturated Fatty Acids of Ascites Tumor Cells.

Fat recovered/mg fat-free dry wt	.128 mg	
" " , wet wt	.019 "	
Iodine No.	72	70
Linoleic acid (%)	14.4	13.7
Linolenic " (%)	5.1	4.9
Arachidonic " (%)	4.0	3.9
Pentaenoic " (%)	3.2	3.3

of methyl linolenate and linoleate extends previous work on the inhibition of certain respiratory enzymes by these compounds(2,3). This study has shown that both aerobic and anaerobic glycolysis are also inhibited by the oxidation products of linolenate and linoleate. The observed effects on both respiration and glycolysis may involve a reaction between the oxidation products of fatty acids and sulfhydryl compounds(17). In view of the effects of oxidized fatty acids on the depolymerization of DNA(4) and on the hemolysis of erythrocytes(18), effects other than those involving sulfhydryl groups may be involved.

Analysis of the unsaturated fatty acids has shown that both linolenic and linoleic acids are present in the ascites cells. The oxidation products of both are inhibitory to cellular activities, and the oxidation products of linolenate (3 double bonds) are considerably more inhibitory than are the oxidation products of linoleate (2 double bonds). A similar difference has been noted in the studies of inhibition of bacterial growth(5) and the depolymerization of DNA(4). Within the cells a mechanism must be operative to hold in check the oxidation of these unsaturated fatty acids and the consequent effects of the oxidation products. Thus there is an absence of fatty acid oxidation products which would be inhibitory to cell division in the ascites tumor. Higher unsaturated fatty acids have also been shown to occur in ascites cells and in other tissues(14,15,19). The action of the oxidation products of these acids is not known.

Summary. 1. Extracts of irradiated methyl linolenate and methyl linoleate inhibited respiratory and glycolytic activities of Ehrlich ascites tumor cells. Respiration showed a greater sensitivity than glycolysis to the oxidation products of both esters. 2. The oxidation products of methyl linolenate inhibited both glycolysis and respiration at lower concentrations than those for methyl linoleate. 3. In contrast to the normal tissues of the mouse, the oxidation of tumor cell lipids by aerobic incubation and ultraviolet light could not be demonstrated by the TBA reaction. 4. Analyses of unsaturated fatty acids of ascites tumor cells are presented.

The author is indebted to Dr. K. M. Wilbur for his suggestions throughout the course of the work. The tumor was kindly supplied by Mrs. Elsie Siegel, Montefiore Hospital, N. Y. City.

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Splanchnic Hemodynamics as Influenced by Hepatic Ischemia.* (22055)

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Certain hemodynamic observations made during a previous investigation in this laboratory(1) have shown that hepatic ischemia is followed by a vasodepressor response. The pertinent observations were a marked decrease in arterial pressure accompanied by elevation of portal venous pressure following 1 to 2 hours of complete hepatic ischemia. It was postulated in the preliminary paper that these manifestations could be attributed, in part at least, to hyperemia of the intestinal vasculature. Dilatation in the mesenteric circulation could cause, or contribute to the increase in portal pressure, and secondly, favor splanchnic pooling of blood which would contribute to the fall in arterial pressure.

The purpose of the present investigation was to study the portal circulation following complete hepatic ischemia of 2 hours' duration. Arterial, portal venous, and inferior vena cava pressures were measured with optical manometers concurrently with direct measurement of portal blood flow, so that hemodynamic calculations pertaining to vascular resistance changes could be made.

Methods. Experiments were conducted on 12 mongrel dogs anesthetized with 30 mg/kg of sodium pentobarbital intravenously. A midline incision was made followed by splenectomy. Residual splenic blood was drained into a heparin solution, filtered and reinfused into animals before beginning the experiment. A brass cannula of suitable size was inserted into the splenic vein until tip was near junction with portal vein. This cannula was connected by tygon tubing to a T-tube, one arm connected to a Gregg optical manometer (B), the other to inflow side of a Shipley-Wilson rotameter(2) (D in Fig. 1). The outflow side of rotameter was connected to a cannula introduced into the external jugular vein. A loose ligature was placed around portal vein as close to the liver as possible. This ligature was led out through brass tubing at E. Catheter (F) attached to manometer C was introduced via femoral vein into the inferior vena cava at level of diaphragm. This pressure was taken for hepatic venous pressure in some of the hemodynamic calculations. The other femoral vein was cannulated for infusion of fluids, additional anesthetic, and anticoagulant. Carotid blood pressure was measured with manometer A. The common hepatic artery was freed of investing tissue and nerve fibers, so that an occluding ligature could be passed around it. Care was taken not to interrupt the nerve fibers, and the ligatures were placed so as to lie between nerves and artery. The gastro-duodenal branch was ligated to minimize retrograde flow into the liver during

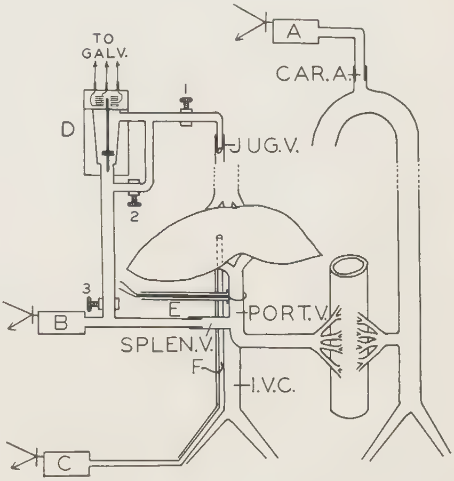

FIG. 1. Details circuit for measuring mesenteric blood flow. A, B, C: Optical manometers for measuring carotid arterial pressure, portal venous pressure, and inferior vena cava pressure respectively. D: Optically recording rotameter. E: Metal tube for ligature which occludes portal vein. Clamp 1 adjusts pressure in external circuit to agree with that registered in portal vein through cannula in splenic vein. Clamp 2 opens rotameter shunt circuit when necessary. Clamp 3 is opened when ligature at E is pulled taut, so that mesenteric blood flow is shunted into external circuit and the rotameter.

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TABLE I. Effect of Hepatic Ischemia on Splanchnic Hemodynamics.

	MABP, mm Hg			Flow, ml/min.†			R_M‡			PVP,§ cm/saline			HR§		
Exp. No.	A*	B*	%	A	B	%	A	B	%	A	B	%	A	B	%
1	116	95	−18	132	148	+12	.81	.58	−28	13.4	13.2	− 1	186	158	−15
2	148	92	−38	219	256	+17	.62	.32	−48	13.8	12.7	− 8	172	124	−28
3	158	138	−13	254	277	+ 9	.58	.46	−20	13.3	13.1	− 2	228	182	−20
4	138	142	+ 3	224	212	− 6	.57	.63	+11	13.7	14.3	+ 4	150	143	− 5
5	153	127	−17	159	161	+ 1	.88	.71	−20	17.8	17.0	− 4	200	177	−12
6	163	136	−17	188	190	+ 1	.85	.68	−20	12.2	12.2	0	209	197	− 6
7	162	150	− 7	207	157	−24	.74	.87	+18	15.7	16.1	+ 3	170	150	−12
8	160	155	− 3	208	191	− 8	.73	.77	+ 6	11.3	12.8	+13	212	190	−10
9	169	151	−11	137	110	−19	1.25	1.28	+ 3	14.8	14.1	− 5	182	160	−12
10	132	114	−14	243	183	−25	.51	.59	+16	12.0	10.5	−13	172	137	−20
11	161	159	− 1	259	262	+ 1	.58	.53	−10	13.2	15.3	+16	162	151	− 7
12	129	116	−10	183	173	− 6	.67	.64	− 5	9.1	9.2	+ 1	153	161	+ 5
Avg			−12			− 4			− 8			+.3			−15

* A, values before ischemia. B, values during final 30 min. of 2-hr ischemia.
† Flow does not include splenic contribution to portal vein.
‡ R_M = Mesenteric resistance, calculated from the ratio: $\frac{\text{MABP} - \text{PVP}}{\text{Flow}}$.
§ PVP = Portal venous pressure. HR = Heart rate.

ischemic period. Prior to beginning of experiment the animals were given a total dose of 40 mg heparin and 85 mg of a heparinoid (Manuronate); maintenance doses were continuously infused thereafter. The following method was used to measure *portal blood flow* preceding and following the period of hepatic ischemia. Portal venous pressure was noted immediately preceding each flow determination. The external circuit was then opened at clamp 3, and the ligature around the portal vein was pulled taut, occluding the vein against the brass tubing, E. The portal blood then flowed through the splenic cannula and the external circuit and rotameter into the jugular cannula. The caliber of tubing in external circuit was adjusted by clamp 1 so that pressure was maintained at exactly the same level as the portal pressure prior to manipulation, *i.e.*, while perfusing the liver. After stabilization, flow and pressures were recorded, then the circuit was closed and blood recirculated through the liver. The above procedure required less than a minute. Records were made at 2-3 minute intervals for an average time of 25 minutes. All flow and pressure measurements were made during the expiratory pause. Zero pressure reference was at the level of the inferior vena cava. No correction was made for intra abdominal pressure under the assumption that it would be approximately equal to the atmospheric pressure with the open abdomen. After initial flow and pressure measurements were made, the hepatic artery was closed and portal ligature at E pulled taut, so that portal blood was continuously diverted through the external shunt circuit for the 2 hour period of ischemia. Pressure in this circuit was kept at approximately the initial portal pressures with the aid of clamp 1. Flow measurements during the period of ischemia were made with continuous flow through the rotameter. Upon release of the occluding ligatures, flow measurements were resumed at first at 1-2 minute, later at 3-5 minute intervals, in the manner used prior to ischemia. Observations were usually continued for about one hour. During and following hepatic ischemia, glucose solution was continuously infused intravenously at the rate of 150 mg/kg/hr with a motor driven syringe.

Results. It is pertinent to present the hemodynamic changes which occurred during the 2 hour period of hepatic ischemia. The information is supplied in Table I, where mean arterial blood pressure, portal venous pressure, heart rate, portal blood flow, and mesenteric vascular resistance during the pre-ischemic period are compared with those obtained in the last half-hour of hepatic ischemia. Portal pressure was maintained at a constant value during the period, but all other functions

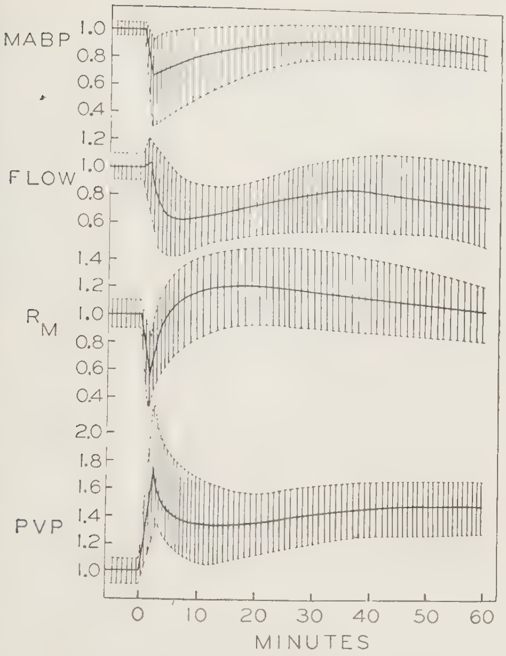

FIG. 2. Splanchnic hemodynamics following 2 hr of hepatic ischemia. Zero time coincides with time of release of ligatures occluding hepatic artery and portal vein. Solid line is avg trend of 8 experiments, expressed as ratios to function during the last 30 min. of the ischemic period. Dashed lines are drawn to indicate range of variation in this group of experiments and encompass *ca* 90% of all observations. MABP: mean arterial (carotid) blood pressure. Flow: portal (mesenteric) flow. PVP: portal venous pressure. R_M: mesenteric vascular resistance, calculated from the ratio $\frac{\text{MABP} - \text{PVP}}{\text{Flow}}$.

showed a tendency to decline by the following average values: arterial pressure, –12%; heart rate, –15%; blood flow –4%; and mesenteric resistance –8%. It was decided for greatest accuracy to compare post-ischemic manifestations with measurements made in the 30 minute period immediately preceding release of occluding ligatures. These values will serve as control values for the experimental changes induced by hepatic ischemia. A total of 8 experiments showed essentially comparable results. These experiments are summarized in Fig. 2 which shows the mean trends following ischemia as ratios to the 30 minute period immediately preceding release of the ischemia.

Mean Arterial Blood Pressure. This decreased rapidly upon release to 66% of the control in 1½ minutes. A gradual restoration of blood pressure followed, and at 35 minutes it had returned to 93% of control. Thereafter, a second downward trend ensued, so that pressure was 86% of control at end of the hour. In several animals which were followed beyond the hour, the downward trend was seen to continue, but in this series none was followed long enough to determine ultimate survival.

Portal blood flow. This showed a momentary tendency to increase during the first 1½ minutes, during which interval blood pressure was rapidly falling. However, flow soon decreased, reaching a value of 63% of control, 6 minutes after restoration of hepatic blood flow. It then gradually increased, attaining a maximal value of 86% of control at 35 minutes. Thereafter it decreased to 75% of control at 60 minutes.

Mesenteric resistance. This showed its greatest decrease 1½ minutes after release, when it was 56% of control, coinciding with the time of most marked decline of arterial pressure. This suggested that the fall in arterial pressure was caused by peripheral vasodilatation in which the mesenteric vessels participated. It is presumed that the drop in arterial pressure initiated compensatory vasomotor reflexes to cause reversal in blood pressure trend. Apparently, participating in compensatory vasomotor reflexes was the intestinal vasculature, for its resistance increased within 15 minutes to 21% above control, during which time arterial pressure showed its maximal rate of recovery. Subsequently, mesenteric resistance decreased somewhat, accompanying the secondary decline in arterial blood pressure.

Portal venous pressure. This increased abruptly on release of the portal vein ligature, attaining a maximal value of 75% above control during the brief period of minimal mesenteric resistance. It remained continuously elevated, however, at a somewhat lower value during remainder of experiment.

Heart rate. This showed no striking change in post-ischemic period, averaging 97% of control for the entire series. One dog, however, decreased to the range 73-87% of control for 14 minutes post-ischemia. In another, heart rate decreased to 85% of control during the interval 4 to 11½ minutes post-ischemia. It is believed, however, that generally heart rate changes are non-contributory to changes in blood pressure. Four animals showed atypical results. One (Exp. 12) never attained a blood pressure higher than 65 mm Hg after ischemia, and died within 15 minutes after release of the ligatures. Portal pressure increased to 18.3 mm Hg, and mesenteric resistance averaged *ca* half the control value during the brief period of post-ischemia. Another (exp. 9) also failed rather soon; 30 minutes after restoration of hepatic circulation arterial pressure started a rapid decline, with concomitant decrease in mesenteric resistance indicating failure of compensation. Two other animals (Exp. 10 and 11) showed continuously increased resistance and reduced blood flow; no phase of reduced resistance could be detected following release of occluding ligatures. Blood pressure was well maintained in both. In these two, either a vasodepressor response was not produced by the liver ischemia or compensation was so rapid and complete as to be undetectable by our method of measurement.

Discussion. The transient phase of reduced mesenteric vascular resistance following restoration of the hepatic circulation attests to the probability that vasodilatation is a contributing component to the initial precipitous collapse of arterial pressure. The extent to which other vascular circuits participate in the response is not presently known. Two alternative mechanisms may be involved: activation of a vasodilator reflex, or release of vasodilator material from the anoxic liver. In consideration of possibilities of a vasodilator reflex, Alexander(3) has noted reflex mesenteric venous dilatation associated with pressure elevation in the inferior vena cava of dogs, but vena cava pressure in the present series showed no significant change following hepatic release. The possibility that increased portal venous pressure would initiate a vascular reflex was investigated(4), but no mesenteric dilatation was observed. Johnson(5) has noted a transient phase of reduced hepatic resistance immediately following transfusion after prolonged hypotension in rats, and has suggested that this may be a dilator reflex in response to elevation in arterial pressure when blood was re-infused. In our investigation, dilatation appeared to be localized in the mesenteric vessels, and concurrently hepatic resistance was increased.

The alternative mechanism, that a vasodepressor substance is released by the anoxic liver, is herewith favored, although based on circumstantial evidence. At present the nature of such a substance is speculative. VDM (vasoactive ferritin) had been proposed by Shorr *et al.*(6) as the vasodilator substance released by liver during anoxia of that organ, but conceivably other metabolites with vasodilator properties might be released in abnormal amounts following hepatic ischemia. Presenting difficulty to the hypothesis that a vasodilator substance is released by the anoxic liver is the fact that the organ itself apparently does not manifest hyperemia after 2 hours of ischemia. But vasomotor responses might be obscured by mechanical obstruction to flow incurred by the ischemia, *e.g.*, edema and swelling of hepatic lobular cells, with compression of the sinusoids.

The coincidence of the phase of reduced mesenteric resistance with the initial period of marked increase in portal pressure demonstrates that this hyperemia contributes to the observed elevation of portal venous pressure. But it is obvious that this is not the only factor—indeed, it may be a minor one—in sustained elevation of portal pressure, which remains elevated while mesenteric resistance is increased and intestinal inflow into the portal vein is reduced. The conclusion follows that the significant factor in elevation of portal pressure following hepatic ischemia is a sustained increase in resistance to portal inflow. If hepatic resistance is calculated from the ratio:

$$\frac{\text{portal pressure—inferior vena cava pressure}}{\text{portal flow}}$$

the ratio increases from 0.026 (.016–.039) (pre-ischemia) to 0.067 (.027–0.114) (post-ischemia) in 6 experiments of this group in which inferior vena cava pressures were measured. The latter pressure is taken as representative of hepatic venous pressure.

The compensatory reflexes which restore arterial pressure following initial collapse probably involve carotid and aortic pressoreceptors. In addition, it has been observed that elevation of portal pressure will itself cause mesenteric resistance to increase(4) by unknown mechanisms. The rapid institution of compensatory mechanisms makes it difficult to assess the true intensity and duration of the hepatic dilator action. The incomplete recovery of arterial pressure despite sustained increase in mesenteric resistance above the control value could be interpreted to mean that the influence of the hepatic depressor mechanism persists longer than the brief period of overt hyperemia indicates. The period of hyperemia could represent a phase of pooling and trapping of blood in capillaries and venules of the intestines (and possibly elsewhere), where it might remain segregated from active circulation, even though compensatory constriction of arterioles had supervened.

Hyperemic responses following anoxia and brief ischemia in other organs is a commonly observed phenomenon, but only a few illustrative examples can be cited here: coronary circulation(7,8), cerebral circulation(9), skin and muscle(10,11). It is generally opined that some metabolic substance having vasodilator properties is released in increased amounts as the result of anoxia to produce hyperemia, but the exact nature of the dilator substance is still a matter of speculation. Possibilities that have been invoked include the adenine nucleotides(12), histamine(10), and acetylcholine(8). When ischemia of the hind limb is prolonged (5-6 hours), renewal of circulation leads to circulatory collapse, the so-called "ischemic compression" shock (for refs. see (13)), presumably as the result of release of vasodepressor substances of a toxic nature by the ischemic limb.

Summary. The influence of complete hepatic ischemia of 2 hours' duration on arterial blood pressure, portal vein pressure, heart rate, and mesenteric vascular resistance was investigated in dogs. During ischemia, small gradual decreases occurred in arterial pressure, heart rate, portal blood flow and mesenteric resistance. On release of the occluding ligatures on the hepatic artery and portal vein, the following changes were observed: an immediate sharp decrease in arterial blood pressure; a brief period of increase in portal blood flow, followed by a decline; a sharp increase in portal venous pressure, and a marked decrease in mesenteric vascular resistance. These manifestations lasted approximately 5 minutes, and were then followed by a partial recovery of arterial pressure and portal blood flow, some reduction of portal venous pressure, and a significant increase in mesenteric resistance. Heart rate showed no significant change.

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Reversal of Fluoroacetate Inhibition by Vitamin B_{12} in a Strain of *Escherichia coli.* (22056)

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The addition of vit. B_{12} to aged cells of *Escherichia coli* strain 113-3, a mutant requiring vit. B_{12} or methionine for growth, stimulates the rate of oxidation of a number of substrates including acetate(1).

A study was conducted to investigate the cause of the stimulatory effect of vit. B_{12}. In the course of the work various metabolic inhibitors were used. Fluoroacetate was employed as an inhibitor of the tricarboxylic acid cycle, since it has been shown to be converted to fluorocitrate which acts as a competitive inhibitor of aconitase(2,3). The data reported here deal with the inhibitory effect of fluoroacetate on acetate and glucose oxidation in this mutant, and its reversal by vit. B_{12}.

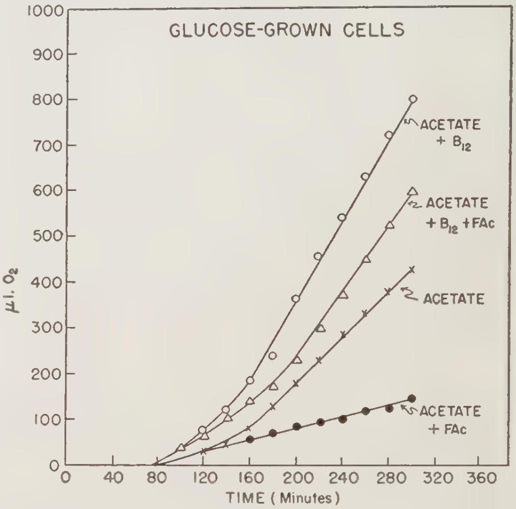

FIG. 1. Acetate oxidation in glucose-grown cells. *Flask contents:* 25 μM acetate, 3 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 1.09 mg. *Citric acid formed:* acetate ± B_{12}, 0; acetate + FAc, 0.1 μM; acetate + FAc + B_{12}, 0.3 μM.

Methods. The medium used was that of Davis and Mingioli(4) containing glucose and supplemented with 20 mg DL-methionine per liter. The organisms[†] were grown at 32°C for 18 hours without aeration in 18 liter pyrex bottles containing 16 liters of medium. The cells were harvested, washed twice, suspended in distilled water and aged in the refrigerator for a minimum period of 10 days. To adapt the cells to acetate, they were transferred in medium in which potassium acetate progressively replaced glucose. The acetate-grown mutant still required B_{12} or methionine for growth. The acetate-adapted cells were grown and treated in the same manner as the glucose-grown cells. The activity of the resting cell suspensions was measured at 37°C using conventional Warburg technics under air. All flasks contained potassium phosphate buffer, pH 7.0 (100 μM); vit. B_{12} (Cobione, Merck) was added at the same time as the substrate and inhibitor. The nitrogen content of the resting cell suspensions was determined by a micro-Kjeldahl method. Citric acid was determined by a modification of the method of Natelson, Lugovoy and Pincus(5).

Results. The effect of fluoroacetate[‡] on acetate oxidation by glucose-grown cells is shown in Fig. 1. Acetate oxidation was inhibited and citric acid accumulated in the presence of fluoroacetate. No citrate was found in the absence of fluoroacetate. The addition of vit. B_{12} stimulated the oxidation of acetate as previously described(1), and decreased the inhibitory effect of fluoroacetate, although it did not affect the amount of citrate formed. The lag in acetate oxidation could be eliminated by the addition of malate. This may indicate that the main cause for the delay in oxidation of acetate is the need for the formation of a C_4-acid which is required for the oxidation of acetate via the tricar-

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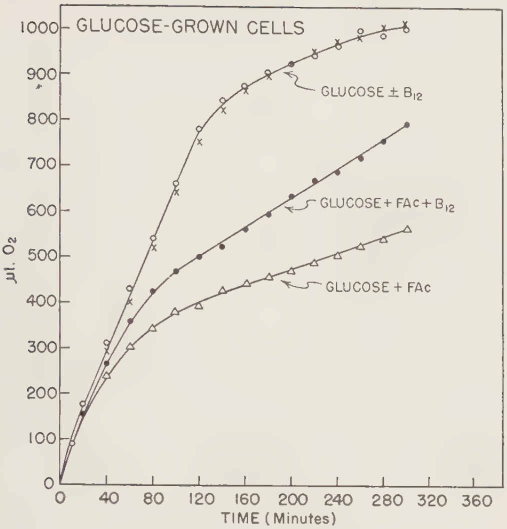

FIG. 2. Glucose oxidation in glucose-grown cells. *Flask contents:* 11 μM glucose, 5 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 1.77 mg. *Citric acid formed:* glucose ± B_{12}, 0; glucose + FAc, 0.3 μM; glucose + FAc + B_{12}, 0.4 μM.

boxylic acid cycle. Vit. B_{12} had no effect on this lag.

Since Ling and Chow(6) reported that vit. B_{12} was involved in glucose oxidation by erythrocytes, the influence of vit. B_{12} and fluoroacetate on glucose oxidation in this mutant was investigated. The results of this study are presented in Fig. 2. Vit. B_{12} had no effect on either the rate or extent of glucose oxidation. A marked inhibition of oxidation resulted from the addition of fluoroacetate, an effect which could be partially prevented by the addition of vit. B_{12} (Fig. 2).

The oxidation of acetate by acetate-grown cells and the effect of fluoroacetate and vit. B_{12} are shown in Fig. 3. No lag was observed in the oxidation of acetate, and vit. B_{12} had no effect on the rate of oxidation. The inhibitory effect of fluoroacetate was not as great as that found in the glucose-grown cells, and the addition of vit. B_{12} completely overcame it. Glucose oxidation was also inhibited by fluoroacetate, but it was only partially reversed by vit. B_{12} (Fig. 4).

The presence of fluoroacetate during the oxidation of glucose and acetate caused the accumulation of citric acid in both the acetate- and glucose-grown cells. Vit. B_{12} had no effect on the amount accumulated. In some cases the amount of citric acid found was slightly greater in the presence of vit. B_{12}. (See Fig. 1, 2, 3, 4).

Discussion. Fluoroacetate decreased the rate of oxidation of acetate and glucose by aged cells of *E. coli,* strain 113-3, which had been grown on glucose or acetate. The extent of inhibition was less in the acetate-adapted cells. Vit. B_{12} prevented this inhibition partially or entirely. The nature of the action of vit. B_{12} is not known. It would be possible to overcome partially the fluoroacetate inhibition by increasing the oxalacetate level or by introducing an alternate pathway of acetate oxidation. In this work malate, which would form oxalacetate on oxidation, did not replace vit. B_{12} in relieving the inhibition. If an alternate pathway is visualized, then acetate oxidation would by-pass the tricarboxylic acid cycle, although fluoroacetate would continue to form fluorocitrate which would accumulate and be determined as citrate. In these studies, although vit. B_{12} prevented some of the decrease in oxidation of acetate by fluoroacetate, it had no effect on the accumulation of citrate. It is unlikely, therefore, that vit. B_{12} affects directly or indirectly the enzyme systems of the tricarboxylic acid cycle.

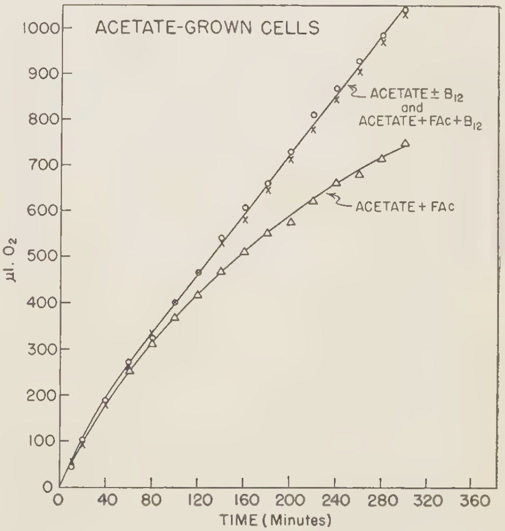

FIG. 3. Acetate oxidation by acetate-grown cells. *Flask contents:* 25 μM acetate, 3 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 0.7 mg. *Citric acid formed:* acetate ± B_{12}, 0; acetate + FAc, 0.6 μM; acetate + FAc + B_{12}, 0.5 μM.

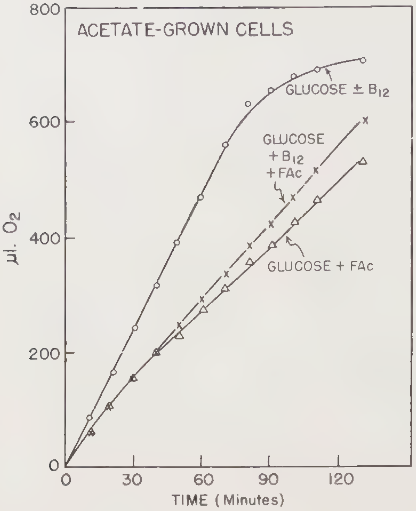

FIG. 4. Glucose oxidation by acetate-grown cells. *Flask contents:* 10 μM glucose, 5 μM fluoroacetate (FAc), 100 mμg B_{12}; N content, 0.7 mg. *Citric acid formed:* glucose ± B_{12}, 0; glucose + FAc, 0.6 μM; glucose + FAc + B_{12}, 0.5 μM.

The alternate pathway may be utilized to a greater extent in acetate-adapted cells, since there is less inhibition by fluoroacetate of acetate oxidation in these cells than in the glucose-grown cells. Vit. B_{12} may function by shifting acetate oxidation into the alternate pathway, either by stimulating the activity of an enzyme system or by stimulating the formation of the enzyme system, or one of its components. Since vit. B_{12} has no effect on acetate oxidation by mutant cells that are not aged, the aging process may cause a destruction or inactivation of some part of the alternate pathway. In acetate-adapted cells in which vit. B_{12} does not affect oxidation, less inactivation must occur.

Thus, as a possible explanation of the influence of vit. B_{12} on fluoroacetate inhibition, it is suggested that vit. B_{12} partially shunts the oxidation of acetate into an alternate terminal oxidative pathway.

Summary. 1. The rate of acetate oxidation in aged, glucose-grown cells of *Escherichia coli* strain 113-3 was stimulated by addition of vit. B_{12}. Stimulation of glucose oxidation was not observed. Upon addition of fluoroacetate with either glucose or acetate as substrate, oxidation was inhibited. This inhibition was partially prevented by the simultaneous supplementation with vit. B_{12}. 2. Acetate-adapted cells of this mutant failed to show a stimulatory effect of vit. B_{12} on oxidation of acetate. However, in the presence of fluoroacetate, oxidation of acetate and glucose was stimulated by vit. B_{12}.

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Effect of Cholic Acid Administration on Taurine Concentration of Rat Organs.* (22057)

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The administration of cholic acid to growing rats causes a suppression of growth if the diet is low in sulfur amino acids(1). This effect can be reversed by feeding cystine, methionine or cysteine, but not taurine. In studying experimental atherosclerosis, Fillios and Mann(2) observed that rats fed cholesterol did not develop hypercholesterolemia if methionine was fed at the same time. White (1) points out that the cysteine deficiency observed after feeding cholic acid is the result of a diversion of cysteine into detoxification reactions. This is a plausible explanation; however, cholic acid is known to form mostly taurocholic acid in the rat(3,4), and endogenous taurine is used up in this reaction. It has been observed that the taurine concentration of rat organs remains constant even when animals are starved(5). Thus taurine must be formed from cystine, cysteine and to a lesser extent from methionine to replace the taurine consumed in the formation of taurocholic acid.

In this paper, data are presented which support the idea that taurine is formed from cysteine (or cystine) even when these amino acids are not supplied in the diet. In such a case, taurine is formed from endogenous cysteine, thus causing a cysteine deficiency.

Methods. Rats of the Sprague-Dawley strain were allowed to gain weight on a diet of Purina chow. When the animals weighed 200-220 g, they were divided in groups of 6 and each animal placed in an individual cage. Food consumption was determined daily. The diets used were prepared from a basic protein-free food mixture† of the following composition:

	%
Cornstarch	70
Vegetable oil	24
Salt mixture U.S.P.I.	4
Cod liver oil	2
Vit. supplement‡	

With this mixture we prepared diets containing 8 and 12% casein. Cholic acid diet was prepared by incorporating in the protein-free diet 8.16 g per kilo of cholic acid. A second diet was prepared containing in addition to the cholic acid 6 g per kilo of taurine. The animals were fed these diets for 10 days. At the end of this period, they were sacrificed and free taurine measured by a method recently reported(5). Liver, kidney, spleen, heart and muscle were studied.

Results. The absence of protein or the presence of either 8 or 12% casein in the diet has no effect upon the free taurine concentration of the organs studied (Table I). In Table II values are given for the taurine concentration of 5 organs from rats fed a protein-free diet and supplemented with cholic acid and cholic acid plus taurine. The values reported in these tables are within the normal range of values which we have reported for fed rats(5). None of the differences shown are significant.

Discussion. The early work of Foster, Hooper and Whipple(6) has already demonstrated that in the formation of taurocholic acid the limiting dietary factor was cholic acid and not taurine. The amount of taurine lost as taurocholic acid is not known precisely. The taurine excretion in the urine amounts to 50 to 80 micromoles per 24 hours(5) (6.2-10.0 mg). It is difficult to assess how much taurocholic acid is formed when animals are fed extra cholic acid in the diet. We believe that sufficient quantities of taurocholic acid are formed to create a deficiency in cysteine/cystine when these are present in limited amounts. The fact that no reduction in the

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* This work was supported by grants from the National Cancer Institute and the American Cancer Society.

† This was obtained from Nutritional Biochemicals.

‡ Vitamin Fortification mixture from Nutritional Biochemicals.

TABLE I. Effect of Dietary Protein on Taurine Concentration of Organs (Female Rats). (Micromoles taurine/g fresh tissue.)

Diet casein, %	Avg wt loss, g	Liver	Kidney	Spleen	Heart	Muscle
12	11	2.0 ± .4	8.3 ± .4	11.5 ± .5	25.0 ± .9	8.4 ± .4
8	14	1.6 ± .1	7.6 ± .4	11.6 ± .5	25.5 ± 1.5	9.2 ± .8
0	30	1.5 ± .2	9.4 ± .2	12.6 ± .4	25.6 ± .4	9.2 ± .7

Values are averages from 6 animals ± stand. error of the mean.

TABLE II. Effect of Cholic Acid Administration on Taurine Content of Rat Organs. (Micromoles taurine/g fresh tissue.)

Diet*	Avg wt loss, g	Liver	Kidney	Spleen	Heart	Muscle
			Males			
Basic (B)	24	1.0 ± .1	6.0 ± .6	12.2 ± .7	21.6 ± 1.4	8.5 ± .7
(B) + Ch	33	1.1 ± .5	6.2 ± .3	13.2 ± .3	27.1 ± .7	11.6 ± 1.3
(B) + Ch + T	30	1.0 ± .2	8.9 ± .5	13.6 ± .7	27.6 ± .9	12.6 ± .7
			Females			
Basic (B)	20	2.6 ± .4	9.1 ± .9	12.5 ± .4	28.1 ± 1.3	9.6 ± .2
(B) + Ch	38	1.8 ± .2	7.9 ± .5	12.0 ± .2	25.5 ± 1.5	7.7 ± .7
(B) + Ch + T	40	1.9 ± .2	10.2 ± .4	13.8 ± .8	30.3 ± .6	10.1 ± .8

* Ch = Cholic acid; T = Taurine.
Values are averages from 6 animals ± stand. error of the mean.

taurine concentration was observed in any of the group studied indicates that taurine was probably formed. If we consider the urinary excretion of taurine alone, the amount of cysteine, or methionine used in 10 days for taurine formation would be the equivalent of 62 to 100 mg. This is a considerable amount. Taurine fed to the animal has no effect in relieving symptoms of cystine/cysteine deficiency. Fed taurine is known to be rapidly excreted(7,8) and it is also possible that the rates at which taurine and cholic acid enter the liver are different. In order for the two to combine, they must be present at the same time at the site of the condensing enzyme. This is not very probable since both compounds differ markedly in properties. In Table II, we show that the addition of an excess of taurine over cholic acid (2:1 on a molar basis) fails to alleviate the effect of cholic acid upon growth, or to cause an increase in the taurine concentration of any of the organs investigated. Thus we must assume that taurine is formed at a fairly constant rate, and that its formation is independent of the amount of taurine present in the diet. Moreover, it can be concluded from this work that dietary taurine is not utilized for taurocholic acid formation to any great extent.

Portmann and Mann(9) have recently reported that taurine-S^{35} administered to rats gives rise to labeled bile salts. The greater portion of taurine, however, was excreted in the urine.

Summary. 1. Taurine concentration of 5 organs was measured in animals fed a protein free diet and diets containing 8 and 12% casein. No changes in taurine concentration were observed in any of the groups. 2. Administration of cholic acid alone or cholic acid plus taurine to rats maintained on a protein free diet had no effect on the taurine concentration of the 5 organs studied.

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Metabolism of Reserpine-C^{14}. II. Species Differences as Studied *in vitro*. (22058)

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The crystalline alkaloid, reserpine, which is currently finding use in the treatment of hypertension(1,2,3) and psychiatric disorders (4,5,6), was identified in extracts from the roots of *Rauwolfia serpentina* Benth by Müller, Schlittler and Bein(7). In a report on the pharmacology of reserpine, Plummer *et al.*(8) mention the wide range in doses necessary to elicit the characteristic tranquilizing response in the various laboratory animals. Since Sheppard, Lucas and Tsien(9) showed that the rat is capable of oxidizing the 4-methoxy carbon of the 3,4,5-trimethoxybenzoic acid (TMBA) moiety to carbon dioxide as well as hydrolyzing reserpine to yield TMBA, it was thought that the ability to degrade reserpine might be related to dose requirements. To test this hypothesis, liver slices of several animal species were tested for their ability to metabolize reserpine. Part of this work was presented at a meeting of the American Chemical Society(10).

Experimental. In vitro: The livers of mature rats, mice, pigeons and guinea pigs were obtained immediately after death from animals sacrificed for the purpose; while those from dogs and rabbits were obtained by biopsy. The livers were sliced freehand and incubated with 200 μg of reserpine-C^{14}. The preparation of fractions as illustrated in Fig. 1, and the methods of counting and synthesis of reserpine labeled with C^{14} in the 4-methoxy carbon of the 3, 4, 5-TMBA moiety are the same as those previously described(9). Subcellular particles of guinea pig liver were prepared according to the method of Schneider (11). Each fraction was incubated with 100 μg of reserpine-C^{14} for 3 hours at 37°C in Krebs bicarbonate buffer. The reaction was stopped with acid and the tissue extracted as previously described(9). *In vivo*: Four male guinea pigs weighing about 400 g were injected with reserpine-C^{14} in vehicle C(9). One animal received 1.0 mg/kg intravenously while the others received 0.5 mg/kg. $C^{14}O_2$ collections were made and counted as previously described(9).

Paper chromatography and radioautography. Aliquots of fraction C and C_B were mounted on Whatman No. 1 filter paper. 40 μg of TMBA were then added to each spot and the sheets chromatographed by descending method with a solvent composed of 5 normal ammonium hydroxide: butanol: isoproponal (3:2:1). Under these conditions TMBA gives an R_F of about 0.61 and fluoresces a bluish purple when exposed to ultraviolet light. To locate the radioactive spots radioautographs were made using Kodak No-Screen X-ray Safety Film.

Results. The results of the *in vitro* studies with liver slices are summarized in Table I. The rat possesses the greatest ability for the oxidation of the 4-methoxy carbon of the 3, 4, 5-trimethoxybenzoic acid moiety to carbon dioxide. Only the pigeon and rabbit seemed to be devoid of this activity. That most of this process depends on oxygen is obvious from the drop in $C^{14}O_2$ recoveries that occurred when incubation was carried out under nitrogen. The rat liver slices, however, show little ability for splitting off TMBA. Surprisingly, the guinea pig liver slices show a very marked ability in this respect, hydrolyzing about 80 per cent of the reserpine in three hours. The mouse and rabbit show fair hy-

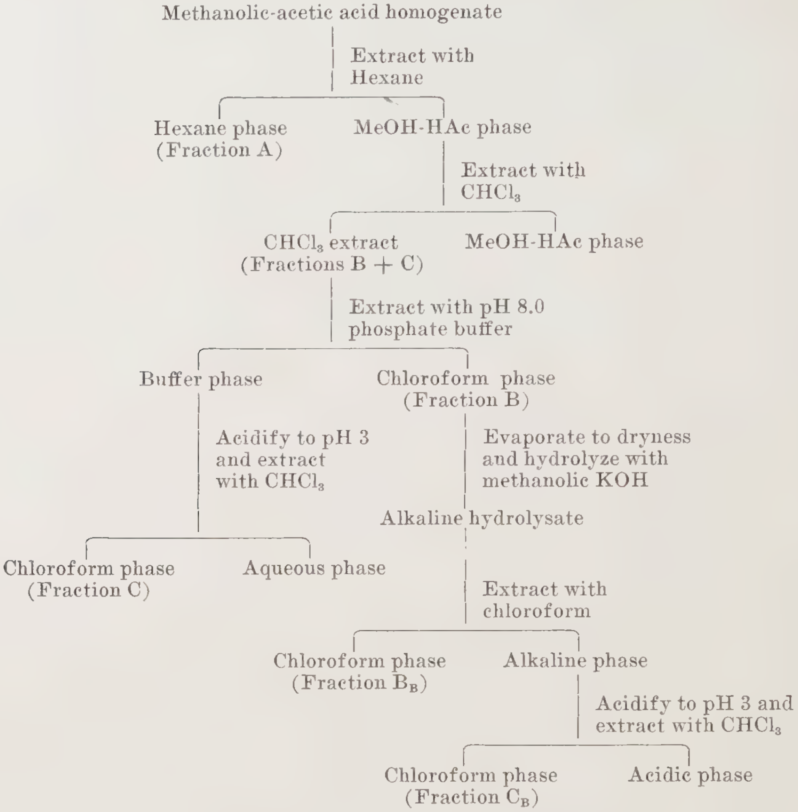
Methanolic-acetic acid homogenate
Extract with Hexane
Hexane phase (Fraction A)
MeOH-HAc phase
Extract with CHCl3
CHCl3 extract (Fractions B + C)
MeOH-HAc phase
Extract with pH 8.0 phosphate buffer
Buffer phase
Chloroform phase (Fraction B)
Acidify to pH 3 and extract with CHCl3
Evaporate to dryness and hydrolyze with methanolic KOH
Alkaline hydrolysate
Chloroform phase (Fraction C)
Aqueous phase
Extract with chloroform
Chloroform phase (Fraction BB)
Alkaline phase
Acidify to pH 3 and extract with CHCl3
Chloroform phase (Fraction CB)
Acidic phase

FIG. 1. Flow sheet for fractionation of liver homogenate.

drolytic activity while the dog and pigeon show practically none. The subcellular particles which possess most of the esteratic activity are the microsomes, as can be seen in Table II. The esteratic activity observed in the other fractions is less, and a portion is probably due to contamination with microsomes.

Radioautographs of paper chromatograms of Fractions C and C_B showed that all of the radioactivity coincided with the bluish purple fluorescence of TMBA.

The urinary excretion of C^{14} by the guinea pig is summarized in Table III. As can be seen, approximately 22% of the injected dose is excreted in the first 24 hours with some C^{14} still being excreted up to 96 hours. As confirmation of the *in vitro* studies indicating a slight ability to oxidize the 4-methoxy carbon of TMBA, some respiratory $C^{14}O_2$ could be detected.

Discussion. Among the animals used in these experiments, the dog is recognized as requiring the smallest and the rat the largest dose of reserpine to produce its typical tranquilizing response. It is apparent from the results presented that the dose requirement of reserpine is not directly related to the ability of liver slices to hydrolyze reserpine nor to demethylate the 4-methoxy group of TMBA or both. If it were, the pigeon would require the smallest and the guinea pig the largest dose of reserpine. The metabolic activity of other tissues must necessarily be considered when studying the response of the whole animal.

Because of the strong esteratic activity present in the guinea pig liver, it was supposed that the guinea pig would rapidly hydrolyze reserpine *in vivo* and excrete the TMBA via the urine. Therefore, it might be possible to demonstrate that pharmacological activity was lost when practically all of the TMBA was excreted, or, if some product of reserpine

TABLE I. *In Vitro* Metabolism of Reserpine-C^{14} by Liver Slices of Different Animals. 500 mg of liver slices incubated with reserpine-C^{14} in Krebs bicarbonate buffer at 37°C for 3 hr. All samples in duplicate.

Species	No. of animals	Atmosphere	% of added C^{14} recovered in fractions				
			CO_2	A*	B†	C‡	Total recovery
Dog	5	O_2	8.51	0	83.4	1.21	93.1
	1	N_2	0	0	96.2	.34	96.5
Pigeon	5	O_2	0	0	89.1	1.25	90.4
	1	N_2	0	0	86.0	1.28	87.3
Rabbit	5	O_2	0	0	80.1	15.7	95.8
	1	N_2	0	0	93.8	10.3	104.1
Mouse	5	O_2	5.6	.15	65.0	31.0	101.7
	1	N_2	—	—	—	—	—
Rat	5	O_2	19.7	.10	58.1	1.37	79.27
	1	N_2	1.3	.06	92.8	2.09	96.25
Guinea pig	5	O_2	2.67	1.51	3.19	80.6	87.97
	1	N_2	3.08	.62	2.25	91.8	97.75

* Fraction A—Contains fat-like substances.
† " B— " reserpine-like substances.
‡ " C— " trimethoxybenzoic acid-like substances.

were responsible for the pharmacological activity, such activity would continue beyond the time when all the TMBA were excreted. The results presented here not only do not support the latter supposition but also demonstrate that hydrolysis does not occur nearly as rapidly *in vivo* as *in vitro*. The reason for this great difference is probably that the reserpine is taken up by tissues which are not very active in metabolizing it(9).

As pointed out previously(9), the increase in the reserpine-containing fractions of rat liver slices incubated under nitrogen, indicates that demethylation probably occurs while TMBA is still esterified with methyl reserpate rather than after hydrolysis has occurred. This was confirmed again with the rat and also found to occur with dog liver slices.

The preponderance of esteratic activity found in the microsomes agrees with the findings of workers concerned with other esterases (12,13). Whether this esterase is a general or specific one remains to be determined.

The radioautograms demonstrate that the C^{14} content of fractions B and C reside in the TMBA moiety. It must still be demonstrated that the alkaloidal portion of the TMBA ester in fraction B is methyl reserpate. Thus far, paper chromatographic or electrophoretic studies fail to distinguish clearly between such alkaloids as reserpine, deserpidine or syringoyl methyl reserpate, indicating that small changes in the reserpine molecule would be extremely difficult to detect by this method.

TABLE II. Hydrolysis of Reserpine by Subcellular Particles of Guinea Pig Liver.

Fraction	% of added C^{14} present in fractions		
	B	C	Total
Homogenate	8.1	83.3	91.4
Nuclei	29.1	58.6	87.7
Mitochondria	35.3	52.5	87.8
Microsomes	9.0	91.9	100.9
Supernatant	10.6	75.7	86.3

TABLE III. Excretion of C^{14} in Urine and Feces of Guinea Pigs after Single Intravenous Injection.*

Rat No.	Interval, hr	Urine		Feces	
		B†	C‡	B†	C‡
1	1-24	.15	8.69	.68	.82
	24-96	.0	.35	.0	.0
2	1-24	.16	7.07	.58	.61
	24-96	.13	.43	.0	.0
3	1-24	.95	4.17	1.32	—
	24-96	.0	.83	.0	.0
4	1- 6	.73	21.9	1.62	1.66
	6-24	.13	1.13	.85	.15
	24-48	.07	.30	.26	.0

* Rats No. 1-3 received 0.5 mg/kg while No. 4 received 1.0 mg/kg.

† Fraction B—Contains reserpine and reserpine-like substances.

‡ Fraction C—Contains TMBA and TMBA-like substances.

Summary. 1. Liver slices of rat, mouse, dog, guinea pig, rabbit and pigeon were incubated with reserpine-C^{14}. 2. Demethylation of the 4-methoxy position of trimethoxybenzoic acid moiety of reserpine was observed to occur, with rat liver slices most active in this respect. 3. Hydrolysis of reserpine was observed to occur with liberation of free trimethoxybenzoic acid. Guinea pig liver slices were most active in this respect. 4. Esteratic activity resides chiefly in the microsomes of guinea pig liver. 5. Demethylation or hydrolysis or both by liver slices is not correlated with dose requirement for typical tranquilizing response. 6. Rate of excretion of C^{14} by the guinea pig was studied *in vivo* after intravenous injection of reserpine-C^{14}.

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Delaying Action of Gonadotrophins on Ovulation in the Hen.* (22059)

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The ovulation-inducing hormone (OIH) in the hen is probably the luteinizing fraction (LH)(1-3), although this action of LH independently of the follicle stimulating hormone (FSH) has not been demonstrated in either birds or mammals(4). As in other spontaneously ovulating animals such as the rat(5) and cow(6), OIH release in the hen is apparently effected over nervous pathways (7-12). The interval from OIH release (or from neural excitation for OIH release) to ovulation is estimated to be not more than about 8 hours(11-15), although it may be greater in some hens(10). A number of pharmacological agents which block LH release in the rat(5,16) were shown recently to suppress ovulation of the first (or C_1) follicle of the hen's cycle following a single injection 38 hours before expected ovulation(17). The agents included anesthetic [diallylbarbituric acid (Dial), pentobarbital Na (Nembutal), and Phenobarbital Na], anticholinergic (atropine sulfate) and antiadrenergic (SKF-501 and Dibenamine) drugs. It seemed highly improbable that some, at least, of these drugs (*e.g.*, atropine or Nembutal) could directly block the OIH release mechanism 30 hours following their injection. Suppression of ovulation was attributed instead to disruption of nervous controls over pituitary gonadotrophin secretions required for maintenance and maturation of the ovarian follicle. The prevalence of follicular atresia in hens injected 38 hours before expected C_1 ovulation and sacrificed shortly after the hour of normally expected ovulation(18) added weight to this conclusion. Ovulation of the second or C_2 follicle was also suppressed by the agents enumerated above, and again in certain instances, under conditions which pointed to disruption of gonadotrophin secretion for maintenance or maturation of the follicle rather than to simple "blockade" of OIH release(18 and unpublished observations).

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* These results were presented at the 44th Annual Meeting of the Poultry Science Assn., East Lansing, Mich., Aug. 9-12, 1955.

One obvious approach to the problem thus posed was to administer gonadotrophins in the expectation that these might counteract the drug action. An anterior pituitary (AP) preparation was accordingly given intramuscularly shortly before injection of representative "blocking" agents. Contrary to expectations, the AP preparation appeared to enhance the suppressing or delaying action of the drugs. These observations led to the suspicion that the AP preparation itself might delay ovulation. This proved to be so. Conceivably, any of several hormones presumably carried by the whole AP preparation might have accounted for its delaying action. Not exhausting all these possibilities, it was nevertheless found that appropriate combinations of LH and FSH could duplicate the effects of the whole AP preparation. In a word, pituitary gonadotrophins may serve in the hen as highly effective "blocking" agents.

Materials and methods. Hens of White Leghorn and crossbred (White Leghorn x Rhode Island Red) extraction served in all but a few test groups; some Rhode Island Red hens were used in these latter groups. The battery caged birds were under artificial illumination from 6:00 a.m. through 8:00 p.m. and had access to feed and water *ad libitum*. Practically all hens were ovulating in 3-day "closed" cycles, ovulating, that is, consecutively on each of 2 days, failing to ovulate on the third day to complete one cycle, and then ovulating on the next day to initiate another cycle(11,12). Typically, the first or C_1 ovulation in the 3-day cycle occurs at about 6:00 a.m., the second or C_2 at around 10:30 a.m. of the following day. The interval between C_1 and C_2 ovulations is thus approximately 28.5 hours. Since ovulation fails to occur on the third day of the cycle, but does so at 6:00 a.m. of the day thereafter, the interval between the C_2 ovulation of one cycle and the C_1 ovulation of the succeeding cycle is some 43.5 hours. All injections were timed for effect on C_2 ovulations. Two measures of response are recognized. The first is simply the incidence of suppressed ovulations, regardless of the fate of the follicle whose ovulation was prevented. The second is the incidence of C_2 ovulations which were delayed only from the normally expected hour of one day (ca. 10:30 a.m.) until early morning hours (ca. 6:00 a.m.) of the following day. In this type of delay the follicle whose ovulation was prevented subsequently behaves like the C_1 follicle of the naturally occurring closed cycle. Such delayed ovulations are therefore designated "C_1 lapses", or merely "lapses". The induced C_1 lapse is identical with the "1-day delay" in ovulation noted elsewhere(11), and is a special case of the "C_1" type" of delayed ovulation first recognized by Rothchild and Fraps(19). Theoretically, the C_1 lapse can be induced only if the specific follicle whose ovulation was prevented is also the follicle subsequently to ovulate. Autopsy findings confirmed this important expectation. The appearance of the C_1 lapse may therefore be accepted as evidence that normally expected OIH release was prevented or failed to occur despite the presence of an ovulable follicle. The occurrence of normal ovulation, of the C_1 lapse, or of longer delays in ovulation was established by palpation procedures described earlier(11,20). Percentages of suppressed ovulations or of C_1 lapses appearing in the Tables are based on the total number of injected hens in each group. Despite care in selection of the birds, an occasional hen will fail to ovulate as expected. A very low incidence of suppressed ovulations or of C_1 lapses (<10 or 20%) is therefore of little or no significance. The "L/S ratio," expressed in percent, gives the proportion of C_1 lapses included in the total of suppressed ovulations. Since follicular atresia is ruled out in the C_1 lapse, but not necessarily in the case of ovulations delayed for greater intervals (2 days or more), the L/S ratio serves as an index of relative follicular immunity to indicated experimental procedures. The "blocking" agents were injected into the muscles of the

TABLE I. Effect of Male Chicken Anterior Pituitary (AP) on Incidence of Delayed C_2 Ovulations following Administration (4:00-5:00 P.M.) of Several "Blocking" Agents.

Injected agents	No. hens inj.	Suppressed ovulations No.	%	C_1 lapses No.	%	L/S ratio (as %)
Atropine	12	10	83	5	42	50
AP + atropine	11	10	91	8	73	80
Nembutal	15	8	53	5	33	63
AP + nembutal	9	8	89	8	89	100
Phenobarbital	11	10	91	1	9	10
AP + phenobarbital	11	11	100	2	18	18

leg at the following dosages: atropine sulfate, 80 mg/kg body weight, Nembutal, 60 mg/kg, and phenobarbital sodium, 100 mg/kg. The drugs were taken up in distilled water immediately before use, one ml water carrying the mg/kg equivalent. Some deaths resulted from the intramuscular injection of Nembutal at the 60 mg/kg level, although none had been encountered when the same dosage was given subcutaneously in other experiments(17).

The AP preparation consisted of anterior pituitary glands from adult Rhode Island Red male chickens. Upon removal, the glands were crushed between glass plates, air dried and subsequently powdered. The powder was suspended in distilled water, 2 or 4 mg/ml, usually shortly before injection into the pectoral muscles; a few suspensions were carried (under refrigeration) over several days. When used with "blocking" agents, the AP preparation was injected some 30-40 minutes before administration of the drugs. The LH and FSH fractions were prepared from anterior pituitary glands of sexually mature male chickens by procedures previously described in the fractionation of sheep pituitary tissue (21). Shortly before injection the fractions were dissolved, separately or together, in distilled water in concentrations which required the administration of not over 1 ml/hen. The solutions were injected into the pectoral muscles. Progesterone was dissolved in corn oil (Mazola), 5 mg/ml, and was injected into the pectoral muscles, 1 mg/hen.

Results. i. Effects of AP on drug action. Examples of drug-induced suppression of C_2 ovulation and AP effects on the drugs are given in Table I. Atropine alone or phenobarbital alone suppressed a high proportion of expected ovulations, yet AP appeared to intensify—however slightly—the suppressing action of the drugs. Nembutal alone suppressed only 53% of expected ovulations, leaving a wider margin for display of any enhancing action by AP. Significantly, AP with the drug increased the incidence of suppressed ovulations from 53 to 89%. The incidence of C_1 lapses increased appreciably when AP was used with atropine (from 42 to 73%) or with Nembutal (from 33 to 89%). With phenobarbital the AP effect was negligible, though in the direction of increased incidence. A high proportion of ovulations suppressed by AP + atropine subsequently exhibited C_1 lapses (L/S = 80%), and all ovulations suppressed by AP + Nembutal did so.

ii. The effects of AP alone. Preliminary tests with AP at several dosage levels indicated that 2 mg/hen, injected at about 4:00 p.m., yielded a fairly high incidence of sup-

TABLE II. Incidence of Suppressed Ovulations and C_1 Lapses following Intramuscular Injection of AP, 2 mg/Hen, at Differing Times before Expected C_2 Ovulation.

Time of inj. (p.m.)	Inj. to ovulation (hr)	No. hens inj.	Suppressed ovulations No.	%	C_1 lapses No.	%	L/S ratio (as %)
1:30	21	7	5	71	4	57	80
4:30	18	22	20	91	18	82	90
6:30	16	12	8	67	4	33	50
11:30	11	7	0	—	—	—	—

TABLE III. Incidence of Delayed C_2 Ovulations following Intramuscular Injection (ca. 5:00 P.M.) of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Combinations of the Fractions.

Quantities LH, mg	Quantities FSH, mg	No. hens inj.	Suppressed ovulations No.	Suppressed ovulations %	C_1 lapses No.	C_1 lapses %	L/S ratio (as %)
.005	—	11	2	18	2	18	100
.01	—	11	4	36	2	18	50
.02	—	12	7	58	4	33	57
.04	—	14	6	43	5	36	83
—	1.0	9	1	11	0	—	—
—	2.0	21	7	33	6	29	86
.02	.5	14	8	57	7	50	88
.02	1.0	27	21	78	16	59	76
.02	2.0	14	13	93	13	93	100

pressed ovulations. This level was used in all experiments summarized in Table II, which in part were carried out to ascertain variation of effect with hour of injection. Despite the relatively small number of hens injected at 1:30 p. m., the results leave little doubt that AP effectively suppressed ovulation when administered as much as 21 hours before the expected event. Four of the 7 treated hens (57%) exhibited C_1 lapses, or 80% of the 5 in which ovulation was suppressed. Following 4:30 p. m. injections, ovulation was suppressed in 20 out of 22 hens. Eighteen of the 22 injected hens (82%), or 90% of the 20 in which ovulation was suppressed, exhibited C_1 lapses. Ovulation was suppressed in 67% of 12 hens injected at 6:30 p. m., but C_1 lapses occurred in only 4 or 33%. As was anticipated from earlier experience, injection of AP at 11:30 p.m. failed to delay ovulation in any of 7 hens.

iii. Effects of LH, FSH and LH + FSH. As can be seen in Table III, LH suppressed few ovulations at the 0.005 mg level, and no large proportion at any of the dosages administered. The incidence of C_1 lapses was relatively low at the 0.02 mg level (33%), which suppressed ovulation in 58% of 12 injected hens. At the 0.04 mg level the proportion of suppressed ovulations dropped somewhat, although the incidence of C_1 lapses increased slightly; the L/S ratio increased from 57 to 83%. Administration of FSH alone at the 1.0 mg level was without appreciable effect in suppressing ovulation. The moderate incidence of suppressed ovulations following injection of FSH at the 2 mg level might possibly be attributed to contaminating LH, and is discussed later. In combining LH and FSH, LH at the 0.02 mg level was injected with increasing levels of FSH (Table III). The incidence both of suppressed ovulations and of C_1 lapses is seen to increase with increasing FSH levels, though at the 1 mg level of FSH the proportion of C_1 lapses did not keep pace with that of suppressed ovulations. This was reflected in the L/S ratio, which came to 76%. The combination of 0.02 mg LH + 2 mg FSH suppressed ovulation in 13 of 14 injected hens, and all of these exhibited the C_1 lapse.

iv. Progesterone effects. Twenty hens were injected with AP (2 mg/hen) at about 4:30 p. m. for delay of C_2 ovulation. The same hens received progesterone (1 mg/hen, intramuscularly) at about 2:30 a. m. of the following morning, *i.e.*, at about the hour of normally expected OIH release. Fifteen of the 20 hens (75%) ovulated either normally or in response to progesterone. This result is to be compared with the effect of AP alone (4:30 p. m. injections, Table II); here only 2 of 22 hens (9%) ovulated at the normally expected hour. It is to be noted that the incidence of progesterone-induced ovulations in AP treated hens is of the same order as was the incidence of C_1 lapses following the administration of AP alone.

Discussion. When injected intravenously, 0.001 mg/hen, 14 hours before expected C_1 ovulation, the same LH fraction used in the present experiments induced ovulation prematurely in some 50% of treated birds(3). The

FSH fraction was about equally effective at the 0.5 mg/hen level. This quantity of FSH (500 times the LH dose) presumably carried sufficient LH as a contaminant to account for the induced ovulations. The moderate effectiveness of FSH in delaying C_2 ovulation (Table III, 2 mg/hen) probably represents, in part at least, the action of LH. If this is so, we may conclude that FSH in itself has little or no capacity to delay C_2 ovulation, but that it substantially increases the potency of LH in this respect. The increased effectiveness of LH in combination with FSH observed here is apparently another instance of the augmentation effect so often encountered when the two fractions are combined(22,23).

Incomplete or partial stimulation of the ovulable follicle by subovulatory doses of LH may result in atresia(24) and thus "suppress" its normally expected ovulation. Van Tienhoven(25) has shown recently that interruption of progesterone-induced OIH release may likewise cause atresia of the ovulable follicle. Interruption of normal OIH release would presumably have the same effect. In the present experiments, atresia of the ovulable follicle might have occurred (though not necessarily), in any hen which failed to exhibit the C_1 lapse. Under appropriate conditions, however, gonadotrophins are capable of inducing the C_1 lapse in practically all injected birds. It is accordingly this characteristic effect which calls for examination and, if possible, explanation.

A first question concerns the immediate source of the suppressed ovulation appearing in the C_1 lapse. The fact that the suppressed C_2 ovulation is typically delayed only until the approximate hour of C_1 ovulation suggests failure of nervous stimulation for normally expected OIH release, such stimulation occurring at the outset of the next diurnally recurrent period of high neural sensitivity(11, 12). The response of gonadotrophin treated hens to progesterone, which causes OIH release by stimulation of the neural component of the release mechanism(7-10,12,25), confirms this inference. We may then conclude that the gonadotrophin-induced C_1 lapse makes its appearance in consequence of a transient "blockade" of the nervous activity required for OIH release.

There are no grounds for supposing that gonadotrophins might act directly as "blocking" agents (*e.g.,* like central depressants). On the contrary, we should expect them to act directly and perhaps exclusively upon the ovary and its secretory functions(4). The causes of gonadotrophin-induced lapses must then be sought in modified concentrations of circulating hormones of ovarian origin.

In appropriate combinations, LH and FSH would be expected to favor estrogen production in the hen, as they certainly do in the rat(26,27). Although estrogen may cause ovulation in the rat and other polyestrous mammals(4,28 for references), it delays ovulation in both the ring dove(29) and the hen (11). The proportion of C_2 ovulations suppressed by estradiol benzoate was only moderate in the hen (about 50%), but some 90% of these subsequently exhibited the C_1 lapse. Moreover, estrogen apparently induced C_1 lapses by increasing threshold requirements in the neural component of the OIH release mechanism(11). In all important respects, then, estrogen and gonadotrophin effects are similar. The gonadotrophin-induced lapse may thus have its origin, in part at least, in elevated levels of circulating estrogen.

Despite the similarity of qualitative actions, gonadotrophins appear to be more effective than either estradiol benzoate or diethylstilbestrol in delaying C_2 ovulation. This may mean that the endogenous estrogen is more "potent" than are the injected substances, or that it is secreted in a fashion not reproduced by injection. There remains, however, another possible aspect of endogenous estrogen production which may not be encountered when estrogen is injected. The gonadotrophin enforced elaboration of estrogen may be associated with delayed or decreased production by the ovary or the maturing follicle of the hormone—possibly a progestin(11,12)—believed to stimulate the neural component of the OIH release mechanism. In this event, gonadotrophin-induced lapses may stem in part from subthreshold levels of the postulated circulating excitation hormone.

If exogenous gonadotrophins result in an altered balance of ovarian hormones (estrogen and progestin), it is not impossible that the naturally occurring OIH release—whether this consist of FSH and LH or LH alone—may do the same. Such a recurrent periodicity in ovarian hormone levels might well play an important part in the determination of lag relationships, length of cycle, and time of onset of the period of lapse(11,12). There is little point in carrying these speculations further at this writing, but they may serve to underscore the very real need for identification of the sex steroids of the ovulating hen and determination of varying concentrations with reference to critical events in the cycle.

The experiments described in this paper do not tell us much about the manner in which the usual nervous "blocking" agents delay ovulation when the delay (or suppression) cannot be attributed to direct blockade of the neural component of the OIH release mechanism(17,18). The increased proportions of C_1 lapses effected by the AP preparations in Nembutal or atropine treated hens may well signify. however, some amelioration of drug action by gonadotrophins or a gonadotrophin. Experiments looking toward a clarification of these relationships are in progress.

Summary. Antiadrenergic, anticholinergic or central depressant drugs suppress or delay, in varying proportions, ovulation of the second or C_2 follicle of the hen's sequence following their administration some 18-20 hours before normally expected ovulation. In an attempt to counteract these effects, a whole anterior pituitary (AP) preparation from cocks was injected intramuscularly shortly before injection of several of the drugs. The incidence of suppressed ovulations was increased by the AP preparation, and increased proportions of suppressed ovulations exhibited C_1 lapses, *i.e.,* the normally expected C_2 ovulation was delayed only until the hour of typical C_1 ovulation on the day following its suppression. Intramuscularly injected 18-19 hours before expected C_2 ovulation, the AP preparation alone suppressed ovulation in most hens, with a high proportion of C_1 lapses. Under similar conditions, luteinizing hormone (LH) or follicle stimulating hormone (FSH) from cock pituitaries suppressed ovulation in some hens. Administration of 0.02 mg LH + 2 mg FSH/hen suppressed ovulation in 93% of injected birds, and all of these exhibited the C_1 lapse. Progesterone injected at about the hour of normally expected release of ovulation-inducing hormone (OIH) effected ovulation in most hens previously treated with the AP preparation, indicating that gonadotrophin-induced lapses resulted from a transient "blockade" of the nervous mechanism controlling OIH release. Since estrogens similarly "block" C_2 ovulation in the hen, the gonadotrophin-induced delay may be attributed to increased levels of circulating estrogen. Enforced estrogen production may however be associated also with reduced or delayed output of the hormone believed to excite the nervous component of the OIH release mechanism.

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Effect of Temperature and Humidity on Nasal Flora of Mice. (22060)

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The possibility that atmospheric conditions are reflected in changes in physiological status of the nose and nasopharynx which are related to susceptibility to upper respiratory infections and poliomyelitis have been suggested. Thus, Armstrong(1) observed a correlation between absolute humidity and incidence of poliomyelitis in certain localities and suggested that atmospheric humidity influences susceptibility by its action on the nasopharynx.

In the present investigation an attempt was made to demonstrate local alterations in normal microflora of nasal passages of mice after exposure to different atmospheres of temperature and humidity, preliminary to a study of association of these changes with local resistance.

Material and methods. White Swiss male mice (9-12 g) were used. Atmospheres of low and high humidity were prepared in metal chambers with glass covers in which cages containing the mice were placed. A low relative humidity in range of 25-30% was maintained by introducing dry air into an enclosed chamber and measured directly by means of hydrodial supplied by the Bendix Co. of Baltimore, Md. A high relative humidity was maintained by introducing air saturated with water into the enclosed chamber. Three sets of the experimental chambers were maintained at 21°C, to study the effect of humidity and normal temperatures. One set was maintained at approximately 31°C to study the effect of increased temperature and humidity. Mice were kept at 21°C with a relative humidity of 45-50% for at least 2 to 3 weeks before they were subjected to experimental conditions. They were fed a balanced diet, and every care taken to avoid infection, exposure, or overcrowding. Bacterial flora of the nasal tract was determined by plating nasal washings on blood agar plates. Nasal washings were obtained as follows: Mice were killed by ether. The skin covering anterior part of head was removed after being moistened with ethyl alcohol. The nasal bone covering the nasal passage was lifted using aseptic technic, thus exposing the nasal cavity. Using a capillary pipette, .05 ml of sterile buffered saline was introduced and after drawing it up and down several times, the washing was spread on a blood agar plate using sterile bent glass rod. The plates were incubated at 37°C for 48 hours and the number and type of normal flora were determined. Nasal washings from 20 mice which had been exposed to low humidity were tested for antibacterial action on isolated gram positive and gram negative bacteria.

Results. The effect of temperature and humidity on normal flora of nasal passage was studied by exposing 4 groups of mice to conditions mentioned previously for 3 weeks. The results summarized in Table I indicated

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TABLE I. Effect of Temperature and Humidity on Normal Flora of the Nasal Tract of Mice.

Humidity	50%		25%		85-90%		85-90%	
Temp.	21°C		21°C		21°C		31°C	
	Total No.	% gram positive	Total No.	% gram positive	Total No.	% gram positive	Total No.	% gram positive
	>1000	50	3	66	>1000	90	about 1000	70
	"	90	111	5	"	90	"	75
	"	90	5	40	about 1000	95	200	90
	"	90	36	22	>1000	95	>1000	90
	450	70	102	2	"	90	"	60
	1000	50	33	9	"	95	700	70
	"	65	69	6	"	90	>1000	70
	"	84	45	28	"	90	500	80
	250	60	47	28	about 1000	90	500	50
	>1000	80	95	5	"	90	about 1000	90

a significant reduction in total numbers of bacteria in the nasal tract of mice kept under low humidity (25%) as compared to those kept at normal (50%) or high (85-90%) humidity. In none of the mice kept at low humidity did the numbers of bacteria equal the lowest number observed in any of the mice kept under normal conditions.

At normal or high humidity the flora was predominantly gram positive, whereas under low humidity the reverse was true. This appears to be due to the marked reduction in gram positive flora and whatever the mechanism involved in these changes is, it seems to have its effect primarily in this group. This inhibition was maximum after 3 weeks exposure. Fig. 1 shows typical plates of normal flora of mice under these different conditions. Fig. 2 shows typical plates of normal flora in nasal washings of mice exposed to low humidity for one and two weeks time as compared with organisms obtained from mice under normal conditions. No antibacterial action could be demonstrated in nasal washings by filter disc method.

Discussion. Our results suggest a significant change in the physiological condition of the nasal passage in response to changes in humidity. Changes in temperature did not produce similar effects. However, there were indications that mice kept under high temperature were "stressed" in that systemic responses associated with stress phenomenon could be demonstrated(*2*). Previous work on correlation of temperature changes with upper respiratory infections has been concerned

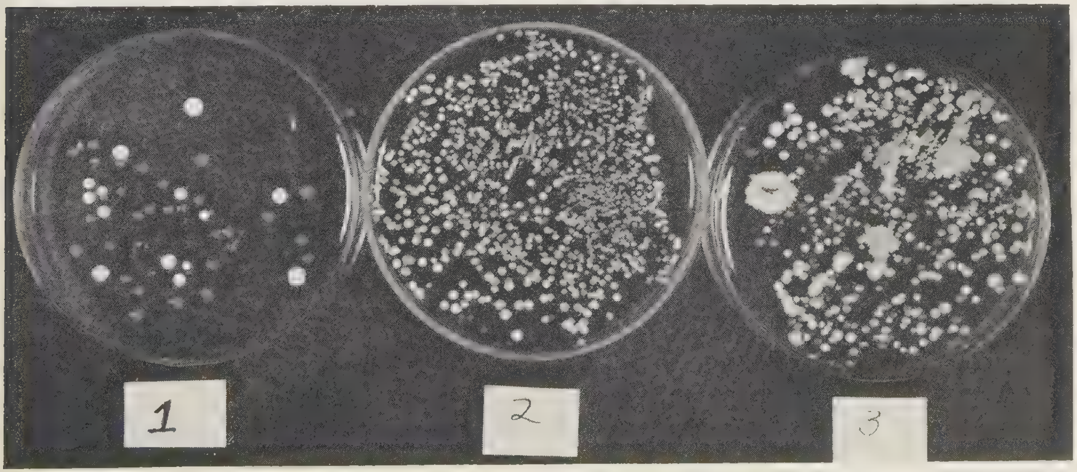

FIG. 1. Plates of nasal washings of mice exposed to the following conditions: 1. Normal temperature (21°C) and low humidity (25%). 2. *Idem*, but high humidity (90%). 3. High temperature (31°C) and high humidity (90%).

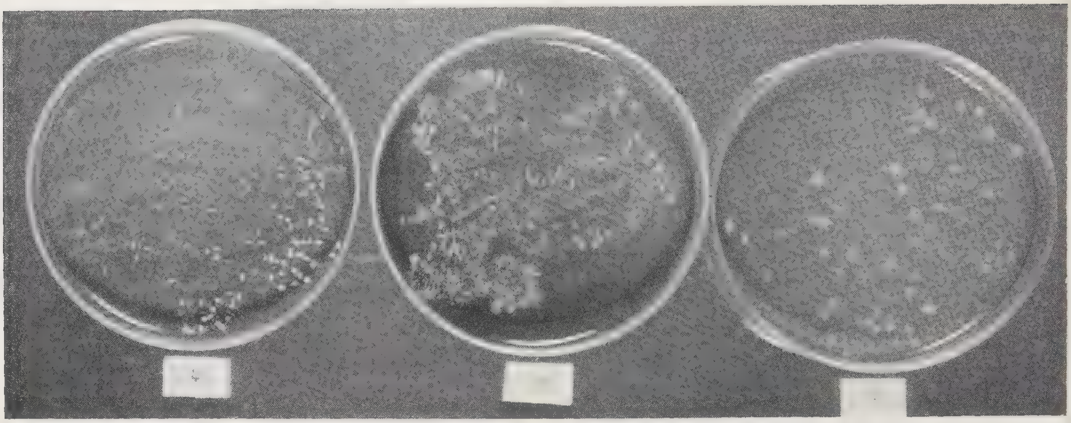

FIG. 2. Plates of nasal washings of mice exposed to normal temperature (21°C) and low humidity (25%) for different periods of time. 4. Control (21°C, 50%). 5. Mice exposed for one week. 6. Mice exposed for 2 weeks.

with the effect of chilling on the physiology of the nasopharynx(3).

Specific antibodies in nasopharyngeal secretions have been demonstrated. Amoss and Taylor(4), Howitt(5), and Bell(6) found specific antibodies against poliomyelitis virus in nasal secretions of a portion of persons examined. Burnet, Lush, and Jackson(7) reported a heat-labile, possibly enzymic substance, in nasal secretions which inactivated certain viruses, notably those of influenza, herpes, and poliomyelitis, but not vaccinia virus. Our failure to show any antibacterial substance in the nasal washings from mice kept at low humidity may be due to the fact that such a substance was present in quantities too minute to be observed by the method used.

Preliminary results have indicated further that conditions favorable for gram positive bacteria in nasal passages appeared to be favorable for infection with influenza virus(2). The reason for the relationship is not clear. It may be that the effect of high humidity on the mucous membrane produced a more favorable environment for gram positive organisms, which in turn affected the mucous membranes in such a way as to make them more susceptible to infection by the virus. On the other hand, it may be that the relationship is more direct and that the change favorable for gram positive organisms also favored the virus. Further work is needed to investigate the association of changes in normal flora of the nasal passage and lowering of local resistance.

Summary. The count of the normal bacterial flora of the nasal cavity was markedly reduced when mice were maintained under low humidity for three weeks, whereas no significant change was observed under high humidity. The reduction was noted mainly in the gram positive organisms.

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Concentration and Distribution of Cholesterol in Muscle and Adipose Tissue.* (22061)

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It is widely assumed that most or all of the cholesterol in muscles, for example in meats, is associated with adipose tissue and interstitial fat and that, in general, with increasing fatness there is an increasing cholesterol content of the whole muscle. This view is reflected in common dietetic practice in which fatty meats are proscribed in low-cholesterol diets, though lean, fat-trimmed meats are allowed.

Actually, however, data have not been available on the distribution of cholesterol between the muscle proper and the associated fat. The present paper summarizes a study on this question with muscle and adipose tissue of 3 species of meat animals, of rabbits, of chickens and of man.

Materials and methods. Beef, veal, pork, suckling pig, mutton, lamb and chickens were purchased fresh in Minneapolis markets. Rabbits were obtained from the University Medical School. Fresh human tissues (abdominal wall muscle and associated adipose tissue and psoas muscle) were provided by the Department of Pathology of the University of Minnesota Medical School. Both commercial and choice grades of round and rib steaks of beef were studied. Leg and loin cuts of veal, mutton and lamb and the longissimus dorsi muscles of rabbit were used. Light (breast) and dark (thigh) meats from roosters, hens and chicks were separately studied. Subcutaneous fat of rabbits, hens and roosters were also analyzed. For each sample the adipose tissue and gross interstitial fat were separated from the lean as completely as possible by careful scalpel dissection. The separated portions were minced in a meat grinder and stored in well-sealed containers at –20°C before analysis. Moisture was determined by drying at low temperature in a vacuum desiccator(1). "Fat" was measured as the weight of the dried ether extract obtained by prolonged extraction in the Soxhlet apparatus. For cholesterol the procedure of Pihl(2) was followed until the chloroform extract was obtained. This was then evaporated, the residue was dissolved in acetone-alcohol, and the cholesterol was precipitated and measured as the digitonide according to the method of Foldes and Wilson(3). Tests of the method gave 98 to 104% recovery of added cholesterol. All measurements were made in duplicate on each sample.

Results. There were no significant differences in the cholesterol results for the different grades and cuts of meat so these are not differentiated in Table I which summarizes 8 independent samples of beef, 4 each of lamb and pork and 2 each for the other

TABLE I. Results from Analyses on Meat. EE = ether extract; cholesterol, mg % EE = mg of cholesterol/100 g of ether extract. Mean values and ± stand. deviations (beef, pork, lamb) or half the range (veal, pig, mutton, rabbit).

	Dissected muscle			Adipose tissue			
						Cholesterol	
Item	EE, %	Water, %	Chol., mg %	EE, %	Water, %	mg %	mg % EE
Beef	5.5 ± 2.3	72.6 ± 3.0	46.8 ± 4.7	75.7 ± 10.9	21.6 ± 9.7	81.5 ± 18.9	104.6 ± 30.0
Veal	1.6 ± .4	74.5 ± 0	71.9 ± 1.0	85.5 ± 3.6	13.6 ± 3.9	76.4 ± 4.3	89.2 ± 1.0
Pork	5.0 ± 1.0	71.0 ± .7	46.4 ± 10.1	90.0 ± 3.9	7.9 ± 3.0	72.6 ± 11.2	80.2 ± 14.8
Pig	3.0 ± .5	76.4 ± .9	83.7 ± .6	68.5 ± 4.3	24.9 ± 3.1	89.3 ± 10.0	121.6 ± 22.4
Mutton	3.0 ± .5	73.7 ± .1	51.8 ± .8	90.2 ± 1.5	6.6 ± 1.2	76.3 ± 6.0	84.4 ± 5.3
Lamb	5.3 ± 2.4	70.6 ± 1.1	60.9 ± 1.3	92.7 ± 4.9	4.7 ± 3.6	67.4 ± 20.0	73.7 ± 26.8
Rabbit	.9 ± .3	74.5 ± 2.8	43.5 ± 1.0	89.2 ± 0	8.5 ± .7	133.7 ± 47.6	149.8 ± 53.3

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* This work was made possible by a fellowship grant from Swift and Co., Chicago.

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TABLE II. Results from Analyses on 5 Patients. Column headings as in Table I.

			Dissected muscle			Adipose tissue			
								Cholesterol	
Case	Age	Sex	EE, %	Water, %	Chol., mg %	EE, %	Water, %	mg %	mg % EE
BS	43	♀	3.4	77.4	65.8	55.5	28.7	150.2	270.5
M	65	♀	22.0	58.9	112.7	96.9	2.3	189.8	195.9
AJ	67	♂	8.3	71.6	138.4	87.4	11.0	323.1	396.6
LB	57	♂	5.6	74.3	59.2	88.4	10.5	95.4	107.9
CM	7	♀	6.6	71.8	64.3	93.9	—	101.1	107.7

items. Table II gives the data from muscle and adipose tissues from 4 adults and a 7-year-old girl. Subjects M and CM had brain tumors, subject BS had carcinoma of the breast, subject AJ had carcinoma of the pancreas, and subject LB had rheumatic heart disease. Table III summarizes the data from chickens. It was impossible to obtain enough adipose tissue from the chick for separate analysis. Inspection of Tables I, II and III shows that, in general, the concentration of cholesterol in the ether extract of the dissected muscle is much higher than in the ether extract of the adipose tissue. However, some of the ether extract obtained from the dissected muscles represents adipose tissue not removed by dissection so some allowance must be made for this. The concentration of cholesterol in such residual adipose tissue should be the same, per unit of ether extract, as in the adipose tissue actually dissected out and analyzed so it should be possible to calculate the amount of cholesterol in the muscle associated with this adipose tissue.

Let E and e represent the grams of ether extract obtained from 100 g of adipose tissue and dissected muscle, respectively, and let K and k be the grams of cholesterol contained in these. Further, let R be the grams of ether extract in 100 g of dissected muscle that is attributable to adipose tissue residue in that muscle, and let C be the grams of cholesterol per 100 g of dissected muscle that is not attributable to residual adipose tissue. Then: 1), $e = R + C$, and 2), $(k\text{-}C)/R = K/E$, so 3), $C = (Ek\text{-}Ke)/(E\text{-}K)$.

Computations from the data in Tables I, II and III are summarized in Table IV. Very little of the cholesterol in the dissected muscle is attributable to residual adipose tissue like that dissected away and it is indicated that truly "lean" muscle contains a very significant amount of cholesterol in all animals, including man.

Further inspection of Table IV shows that this non-adipose tissue cholesterol tends to be higher in the young than in the mature animals of the same species (beef vs. veal, pork vs. pig, mutton vs. lamb). The data do not allow exact calculations for the chicken but in this case, too, it would appear that the rule must hold (Table III). The data from the human subjects are inadequate for the examination of this question and, in any case, may be unrepresentative of normal persons.

Discussion. In most of the previous studies of cholesterol in tissues the Liebermann-Burchard reagent has been applied directly to the non-saponifiable residue from the lipid extraction without separating the digitonide. In

TABLE III. Results from Analyses on Roosters, Hens, and Chicks. L = light meat, D = dark meat. Column headings as in Table I.

		Dissected muscle			Adipose tissue			
							Cholesterol	
Item		EE, %	Water, %	Chol., mg %	EE, %	Water, %	mg %	mg % EE
Rooster	L	.6	72.6	31.7	97.5	4.0	76.4	78.4
"	D	.8	73.1	55.3				
Hen	L	1.5	70.3	31.7	97.3	4.6	91.3	93.9
"	D	4.9	71.1	53.7				
Chick	L	.6	74.6	66.1	—	—	—	—
"	D	1.5	77.3	97.6	—	—	—	—

TABLE IV. Computed Mean Values for Concentration of Cholesterol in mg per 100 g of Muscle Free of Adipose Tissue.

	Cholesterol		Cholesterol
Patient BS	58.8	Beef	43.3
" M	89.4	Veal	71.6
" AJ	117.8	Pork	44.6
" LB	56.4	Pig	82.4
" CM	61.3	Mutton	50.7
Rooster L	31.5	Lamb	60.3
" D	55.1	Rabbit	42.6
Hen L	30.8		
" D	51.6		

the present study this "direct" proceeding was applied in parallel with our digitonin method in aliquots from 34 samples. The "direct" result averaged 16.9% higher (s.d. = ± 10.8%) than the digitonin result so it appears that about a sixth of the non-saponifiable Liebermann-Burchard reacting material in muscle is not cholesterol. This explains, in part, the fact that most of the previously published analyses for cholesterol in meats are higher than those given here(cf, 4-6).

It has been reported that different muscles in the same animal may differ somewhat in cholesterol concentration(7-9), and it has been suggested that these differences are related to differences in the customary activity of the muscles(10-12). It is interesting that red muscles (semi-tendinosus) contain more cholesterol than white (biceps femoralis) muscle in the rabbit(8) and that, as reported here, the same rule holds in the chicken(7).

If to these considerations we add the fact of higher concentrations of cholesterol in the muscle tissue of younger animals it would appear that perhaps there is a general rule for the cholesterol concentration in muscle to be related to the total energy metabolism representing growth of the muscle and muscular work, or both. And it is difficult to avoid the conclusion that, far from being inert, cholesterol has an important role in structure or function of the muscle.

The present calculations for the non-adipose tissue cholesterol in muscle are approximations, of course, because there are certainly other ether-soluble materials in muscle besides cholesterol that are not associated with adipose tissue. Equation 1), above, more properly should be written 4), $e = R + C + X$, where X represents other ether-soluble materials not accounted for by adipose tissue. Presumably, X might include some of the lecithin, for example, found in muscle. But X must be a small quantity compared to R and the error in the estimation of C resulting from its neglect should be trivial.

Summary. 1. Analyses are reported for cholesterol contents of muscle and adipose tissue from beef, veal, pork, pig, mutton, lamb, rabbit, chicken and man. It is shown that a large proportion of the cholesterol in muscle is not attributable to residual adipose tissue or any tissue of similar composition. 2. Concentration of cholesterol in muscle, apart from associated adipose tissue, is of the order of 40 to 50 mg per 100 g in the adult mammals studied and in the dark meat of chickens. Higher values were found in two elderly human beings. The corresponding cholesterol concentration in the white meat of chickens is of the order of 30 mg per 100 g. 3. There is a general tendency for muscle cholesterol concentration to be considerably higher in young, growing animals than in adults of the same species. This reinforces the theory that most of the cholesterol in muscle has a definite metabolic or structural function.

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Cardiac, Renal and Hepatic Hypertrophy in Pyridoxine-Deficient Rats.*
(22062)

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A statistically significant increase in the weight of the heart and kidneys of the pyridoxine-deficient rat was reported by Agnew (1,2). Olsen and Martindale(3) recently confirmed this work and also reported an increase in the weight of the liver of the pyridoxine-deficient rat.

The present study was undertaken to see: (1) if the increases in weight of the heart, kidneys, and liver could be found after feeding the deficient diet for a much shorter period of time than in previous experiments; and (2) if these organs were truly hypertrophied or merely heavier by virtue of increased tissue fluid content.

Methods. Male Wistar weanling rats, obtained from the Charles River Breeding Laboratories, Boston, were used, and were housed in individual cages with wide wire-screened bases. The synthetic diet used consisted of sucrose 72%, casein (Sheffield "vitamin-free") 18%, corn oil (Mazola) 5%, salt mixture(4) 4%, cod liver oil 1%. Choline chloride (1.5 g/kilo) was added, and then appropriate amounts of the following substances in alcoholic solution or suspension to give the following concentrations (mg/kilo) in the complete diet: calcium pantothenate 20, niacin 20, riboflavin 3, thiamin 3, pyridoxine HCl (controls only) 3, folic acid 1, biotin 0.2. Fresh diet was made up weekly and stored in the refrigerator. The animals were weighed daily, and had unlimited access to water. The design of the experiment was similar to that used in previous work(1), and involved the use of 12 trios of rats, each trio (littermates) being arranged thus: Rat 1 received the experimental diet *ad lib.* with all vitamin supplements; 2 received the diet *ad lib.* with all vitamin supplements except pyridoxine; No. 3 was paired-weighed with No. 2 but was given pyridoxine; *i.e.* it received the amount of diet required to keep it equal in weight to No. 2. After 55 days the animals were anesthetized with ether, bled by severance of neck vessels, and the livers, kidneys and hearts dissected out; the hearts were sliced open and any blood removed from the chambers. The organs were washed in 0.9% NaCl solution, gently dried between sheets of filter paper, and the wet weights determined on a Roller-Smith Precision balance. The organs were placed on watch glasses, dried at 58°-60°C for 7 days, and the dry weights then determined.

Results. The results are summarized in Table I. The wet and dry weights of the hearts and kidneys of the pyridoxine-deficient animals were significantly heavier (g/100 g bodyweight) than those of the *ad lib.* and paired-weighed controls. The wet and dry weights of the livers of the pyridoxine-deficient rats, although significantly heavier than those of the paired-weighed controls, did not differ significantly from those of the *ad lib.* controls. Thus it would appear that the increase in weight of these organs is a true hypertrophy and not the result of an increased tissue fluid content in them. Increased fat content was not a factor as histological examination of frozen sections of tissues obtained in earlier experiments(1,2) did not reveal the amount of fat that would have been expected if the increase in organ weight had been caused by increased fat; further, when the dried hearts and kidneys referred to in Table I were defatted, those that belonged to pyridoxine-deficient rats were still heavier than those of the *ad lib.* and paired-weighed controls.

The results obtained (Table I) also show that protracted feeding of the pyridoxine-deficient diet is not necessary to induce hypertrophy of the heart, kidneys and liver as

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TABLE I. Wet and Dry Organ Weights (g/100 g Body Weight) of *Ad lib.* Fed, Paired-Weighed, and Pyridoxine-Deficient Rats.

Organ	*Ad lib.* control (11 animals*)	Paired-weighed control (12 animals)	Pyridoxine-deficient (12 animals)
Body wt	310.9 ±30.6	124.7 ±9.6	130.5 ±10.1
Liver, wet	4.30 ± .66	3.34 ± .27	4.24 ± .45
dry	1.38 ± .08	1.04 ± .09	1.38 ± .18
Kidney, wet	.689 ± .045	.834 ± .069	1.159 ± .138
dry	.166 ± .008	.194 ± .014	.261 ± .030
Dried and defatted	.147 ± .007	.174 ± .012	.234 ± .026
Heart, wet	.307 ± .022	.344 ± .022	.435 ± .068
dry	.069 ± .005	.079 ± .005	.100 ± .017
Dried and defatted	.064 ± .004	.073 ± .005	.092 ± .015

* Originally 12 animals, but one was accidentally killed before experiment ended.

the duration of the experiment was only 55 days. Agnew(1) had previously reported the occurrence of marked cardiac and renal hypertrophy in rats that had been on the deficient diet for periods ranging from 79 to 175 days; and Olsen and Martindale(3) had obtained similar results after 266 days. These latter workers also reported the occurrence of liver hypertrophy in their deficient rats, but in the present experiments the weights of the livers of the pyridoxine-deficient rats, although significantly heavier than those of paired-weighed controls, did not differ significantly from those of *ad lib.* controls.

Discussion. The reason for the significant increase in the weight of such important organs as the heart, kidneys and liver in the pyridoxine-deficient rat is as yet unknown. It is possible that the increased heart weight is secondary to the mild hypertension that has been reported(5) in pyridoxine-deficient rats; also, it may be that the increased kidney weight is the result of the increased excretion of urea that has been described(6) in pyridoxine-deficient rats. Whatever the explanation, pyridoxine deficiency in the rat appears to be another method for inducing cardiac, renal and hepatic hypertrophy, and one that may prove of value in future studies of organ growth and function.

Summary. The wet and dry weights of the heart and kidneys of pyridoxine-deficient rats were significantly heavier (g/100 g bodyweight) than those of *ad lib.* and paired-weighed littermate controls. The wet and dry weights of the livers of pyridoxine-deficient rats, although significantly heavier than those of paired-weighed controls, did not differ significantly from those of *ad lib.* controls. The increased weight of these organs is considered to be a true hypertrophy, and was observed after a period of 55 days. It is felt that this relatively rapid method of inducing hypertrophy of these important organs may prove a useful tool in studies of organ growth and function.

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Effect of Ascorbic Acid on Cysteine Requirement of Lactobacilli.* (22063)

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In a study of amino acid requirements of a collection of oral lactobacilli it was found that, in addition to other amino acids required for growth, cysteine was needed by 23 of 28 oral cultures and also by 3 of 5 American Type Culture Collection lactobacilli(1). This result was obtained with a chemically defined medium containing 18 amino acids including methionine, needed B vitamins, purines and pyrimidines, glucose, acetate, and salts. The proportion of lactobacilli requiring cysteine under these conditions was, in general, similar to the previous result of Dunn *et al.*(2) who found that 8 of 12 lactobacilli (exclusive of other lactic acid bacteria studied) needed cysteine for growth. These lactobacilli were mostly ATCC strains obtained originally from dairy products or fermentations. Other workers also have studied the needs of certain individual strains which, for the most part, were included in the study of Dunn *et al.*(2) and need not be cited in detail here.

TABLE I. Effect of Ascorbic Acid on Cysteine Requirement of Lactobacilli.

Cultures	No. of cultures growing in chemically defined medium*		
	Complete amino acid medium	On omission of cysteine	Ascorbic acid substituted for cysteine
L. casei	12	1	2†
" , lactose neg.	4	0	0
L. plantarum	7	3	3
L. acidophilus	4	0	0
L. fermenti	8	2	6
L. brevis	1	0	0
	36	6‡	11

* The chemically defined medium contained 200 mg DL-methionine and 100 mg L-cysteine • HCl per 1000 ml. The detailed composition has been given elsewhere(1).

† Slow growth of 1 of the 2 cultures.

‡ The 6 cultures which grew on omission of cysteine were: 1 oral *L. casei*, *L. plantarum* ATCC 8014 (*i.e.*, *L. arabinosus* 17-5), 2 oral *L. plantarum*, *L. fermenti* ATCC 9338, and one oral *L. fermenti*. Growth of most of these cultures was stimulated to some extent by added cysteine though cysteine was not essential for growth.

TABLE II. Response of Certain Lactobacilli to Ascorbic Acid and Cysteine.

Culture designation and strain No.		Additions to medium*		Growth,† days			
		Ascorbic acid, .3 mg/ml	Cysteine • HCl, 100 μg/ml	2	3	4	5-7
L. casei	1006	0	0	3	8	11	13
		0	+	78	83		
		+	0	15	27	49	51
		+	+	62	82		
L. fermenti	55	0	0	1	2	2	1
		0	+	86	88		
		+	0	73	87		
		+	+	86	89		
"	1007	0	0	2	2	1	2
		0	+	4	5	6	76
		+	0	11	54	66	
		+	+	9	17		63
L. casei	1009‡	0	0	11		14	15
(lactose neg.)		0	+	80	82		
		+	0	6	10		9
		+	+	78	82		

* The usual synthetic medium minus cysteine was used. + = ascorbic acid or cysteine • HCl added in amount indicated, 0 = not added. Solutions of compounds were sterilized by filtration and added to the medium after autoclaving and just before inoculation.

† Figures for growth represent turbidity. Lumetron readings of % light transmission with a 650 mμ filter were subtracted from 100. This was done for the purpose of showing increasing turbidities by higher figures. 0 = clear tube, 66 to 87 = heavy turbidity, near maximal or maximal growth. Incubation was at 37°C.

‡ Culture 1009 is included for comparison with the others. It failed to grow in the absence of cysteine whether or not ascorbic acid was supplied. It is representative of the 25 strains of lactobacilli which required cysteine whether or not ascorbic acid was present.

Recently, in continuing our study of cysteine requirement it was found that on addition of ascorbic acid to the chemically defined medium, cysteine was no longer required by certain lactobacilli. Others, though still requiring cysteine for growth, showed a more rapid growth rate in the presence of ascorbic acid. These findings are presented here.

Results. Table I gives the result of tests of cysteine requirement and of the substitu-

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TABLE III. Effect of Different Amounts of Ascorbic Acid as Substitute for Cysteine in Growth of *L. fermenti.*

	L. fermenti strain numbers:														
	6					55			1002			1007		
	Growth at days														
Ascorbic acid,† mg/ml	2	3	4	7	10	2	3	7	2	3	7	3	4	5
0	0	0	0	0	0	0	0	0	0	0	0*	0	0	0
.01						0	0	0				0	0	0
.1	0	0	0	0	0	11	81		0	0	51	0	0	0
.3	0	0	0	0	75	63	85		6	59	67	0	19	64
.5									36	58	60	5	38	40
1.0	0	0	36	81		75	87		55	60	55	0	39	60
2.0	55	85				81	84		43	46	40	25	26	23

Turbidity is stated as in Table II.

* Occasional slow growth (40 to 50) appeared in from 7 to 10 days, but more often negative.

† Solutions were sterilized by filtration and added to the medium just before inoculation.

tion of ascorbic acid for cysteine as determined for our collection of lactobacilli. Of 36 lactobacilli which grew in a chemically defined medium containing both cysteine and methionine (but not ascorbic acid), only 6 were able to grow when cysteine was omitted; 30 failed to grow. The distribution of these cultures according to species, as determined in other work(1), is given in the table. In the last column it is seen that when ascorbic acid was supplied 11 cultures (the same 6 plus 5 more) were able to grow without cysteine. That is, in the presence of ascorbic acid a cysteine requirement was no longer exhibited by 5 strains: 1 *L. casei* and 4 *L. fermenti.*

Table II shows the individual responses to cysteine and ascorbic acid of 3 of the foregoing 5 lactobacilli and also, for comparison, of 1 other lactobacillus. All failed to grow when cysteine was omitted from the usual synthetic medium, but grew when 100 μg per ml of cysteine • HCl was supplied, though one of them (1007) always grew slowly. When ascorbic acid was supplied in place of cysteine growth of the first 3 organisms occurred. No. 1006 grew more slowly and not as fully as in the presence of cysteine, 55 responded to ascorbic acid as readily as to cysteine, and 1007 grew slowly when either cysteine or ascorbic acid, or both, was supplied. The needs of this strain for prompt growth are not known. The remaining 2 strains, not shown in Table II, grew promptly when sufficient ascorbic acid was supplied in place of cysteine. For all 5 strains there was an apparent substitution of

TABLE IV. Effect of Some Sulfur-Containing Compounds When Substituted for Cysteine.

S-containing compound	Amt, mg/ml	*L. casei* 1006	*L. fermenti* 55	*L. fermenti* 1007
0	0	2	0	0
Na thioglycolate	.1	75	85	69 (9)
	.3	74	74	73 (9)
	1.0	77 (3)	84 (6)	72 (7)
Mercaptosuccinic acid	.1	75	85 (3)	0
	.3	79	78	41 (6)
	1.0	79 (3)	77	50 (4)
2-Mercaptoethanol	.1	46	0	0
	.3	48	0	0
	1.0	52 (6)	0	0
Na thiosulfate pentahydrate	.1	75	83	0
	.3	74	84	0
	1.0	74 (3)	87 (7)	0

Results are for 48 hr at 37°C unless otherwise noted. Slower growth, when it appeared, is indicated by the day in parenthesis following the turbidity figure.

All tubes were held for 10 to 14 days before discarding.

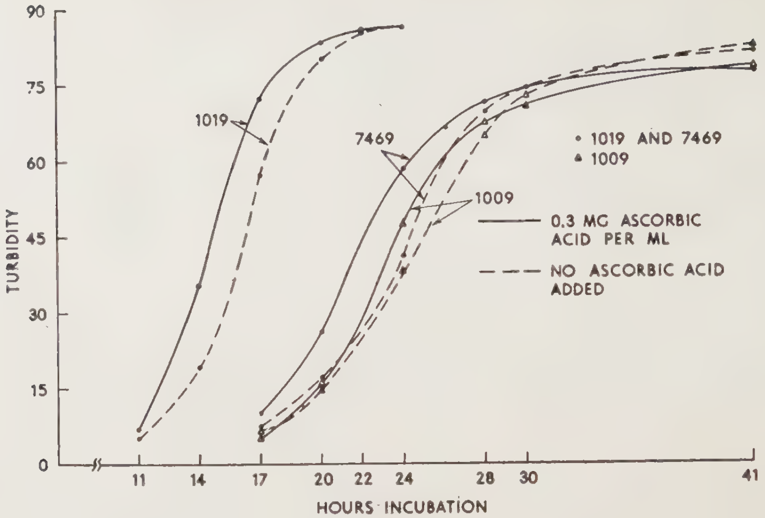

FIG. 1. Growth response of 3 lactobacilli to cysteine and to cysteine plus ascorbic acid. Cysteine • HCl, 100 μg/ml, was added to previously autoclaved medium. The cultures are *L. plantarum* 1019, *L. casei* ATCC 7469, and lactose negative *L. casei* 1009; 1019 and 1009 are of oral origin. These cultures all require cysteine irrespective of presence of ascorbic acid.

ascorbic acid for cysteine, though only partial in one case. These results were verified by repeated tests.

The effect of different amounts of ascorbic acid is shown in Table III. One strain regularly required as much as 2.0 mg per ml of medium for prompt growth while 2 others grew readily in the presence of 0.3 to 0.5 mg per ml. In certain instances there was evidence of some inhibition at the 2.0 mg level.

Other reducing substances were tested as substitutes for cysteine. In a medium lacking both cysteine and ascorbic acid, the cultures responded to additions of sodium thioglycolate or to mercaptosuccinic acid (Table IV). 2-Mercaptoethanol supported moderate growth of only 1 of 3, and sodium thiosulfate supported good growth of 2 of 3 cultures. Evidently ascorbic acid *per se* is not needed and the growth promoting effect can be secured by appropriate amounts of certain other reducing agents, though not all of the compounds are effective for all cultures. The apparent requirement for cysteine on the part of these lactobacilli is evidently a need for a reducing agent and not for cysteine, or ascorbic acid, as a structural entity.

Lactobacilli which require cysteine in the presence of ascorbic acid. Of the 36 lactobacilli studied, there remained 25 cultures which continued to exhibit a cysteine requirement in the presence of ascorbic acid, in contrast to the above cultures. For these 25 cultures cysteine apparently is needed for purposes other than the reducing effect. To determine what effect, if any, ascorbic acid might exert on growth of these lactobacilli in the presence of cysteine, observations of growth were made at different intervals with ascorbic acid added both to optimum and suboptimum amounts of cysteine. Addition of ascorbic acid to a medium containing sufficient cysteine for maximal growth produced a somewhat accelerated rate of growth, but not heavier final growth (Fig. 1). Similar results were secured with all other cultures tested in this manner: 1 *L. plantarum,* 4 *L. casei* of oral origin, and 1 *L. fermenti* which required cysteine in the presence of ascorbic acid.

For experiments with suboptimal amounts of cysteine 4 strains of lactobacilli were tested in the presence of 0, 1, 4, and 10 μg of cysteine • HCl per ml of medium. The last amount is sufficient for maximal growth of some lactobacilli but not enough for others. One set of tubes received 0.3 mg ascorbic acid per ml, the other received no ascorbic acid. It was found that the addition of ascorbic acid

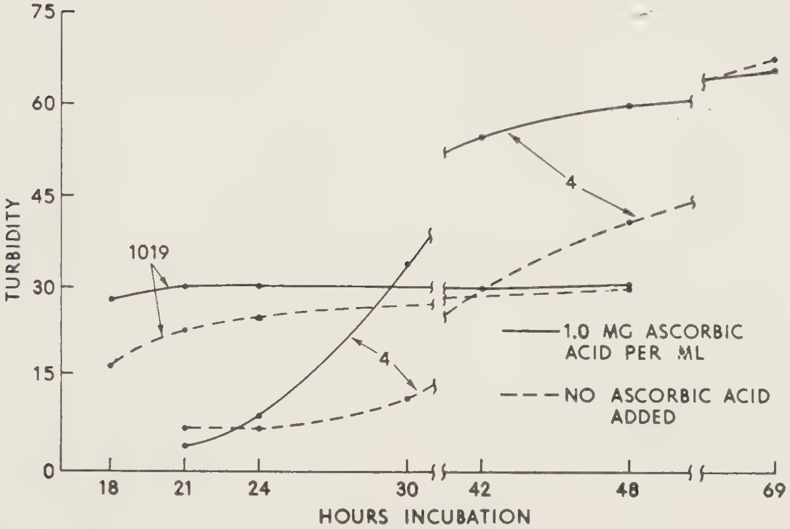

FIG. 2. Growth response of 2 lactobacilli to smaller amounts of cysteine • HCl, with and without ascorbic acid. Strain 4, a representative of lactose-negative *L. casei*, was grown in the presence of 4 μg cysteine • HCl/ml. Growth of *L. plantarum* is distinctly limited by the small amount of cysteine • HCl, 1 μg/ml, but in the presence of ascorbic acid growth is distinctly greater at the earlier data points.

accelerated the rate of growth in most instances, but there was little difference in the final amount of growth if the tubes were incubated a sufficient length of time (Fig. 2). Often several days were required to demonstrate this because growth was somewhat slower in the presence of the smaller amounts of cysteine • HCl.

Discussion. Four of the 5 cultures which do not need cysteine in the presence of appropriate reducing agents belong to the heterofermentative *L. fermenti* species. This seems significant from the standpoint of characteristics of this organism and its distinction from homofermentative lactobacilli. It suggests that a cysteine requirement is actually not as common among *L. fermenti* strains as former results had indicated and that *L. fermenti* differs from most homofermentative lactobacilli in this respect. In our collection of cultures, 6 of a total of 8 *L. fermenti* strains grew without cysteine. Two of the 6 (including the well-known ATCC 9338 strain) were able to grow in the usual chemically defined experimental media without additional reducing agents, but the other 4 grew without cysteine only in the presence of certain reducing agents. The remaining 2 strains required cysteine under all conditions tested. Whether other species of heterofermentative lactobacilli might exhibit deportment similar to the majority of *L. fermenti* cultures is not known since a sufficient number of strains has not been examined.

The ability of *L. fermenti* to dispense with preformed cysteine is in contrast to homofermentative lactobacilli, most of which exhibit a definite requirement for cysteine. In our collection of cultures only *L. plantarum* was somewhat of an exception to this trend in that 3 of 7 strains did not need cysteine. This difference in amino acid requirement is of interest in connection with another such difference recently noted. In previous work(1) it was found that threonine was either a definite requirement or stimulated growth of 5 *L. fermenti* strains, but was neither essential nor notably stimulatory for homofermentative lactobacilli: *L. casei,* lactose-negative *L. casei* and *L. plantarum* (including cultures designated *L. arabinosus* and *L. pentosus*). The present results indicate that the unessentiality of cysteine for most *L. fermenti* cultures under appropriate reducing conditions may be considered as another characteristic of this species, thus bringing to light one more distinction between *L. fermenti* and homofermentative lactobacilli with respect to amino acid needs. A summary of other differentiating metabolic and cultural characteristics and their significance with respect to classification of the genus *Lactobacillus* has been given by Rogosa *et al.*(3).

The results have a possible bearing, too, on assay of cystine by microbiological methods. Not only is the rate of growth of many different lactobacilli accelerated by ascorbic acid in the presence of both suboptimum and optimum amounts of cysteine, but an apparent cysteine requirement of some cultures, mostly *L. fermenti,* disappears in the presence of ascorbic acid or certain other reducing agents.

Summary. 1. The requirement of some lactobacilli for cysteine, as determined in growth tests with usual chemically defined media, disappears when ascorbic acid in appropriate amounts, or other reducing substance, is incorporated in the medium. Of 5 strains of lactobacilli in this category encountered in a collection of 36 cultures, one was *L. casei,* the other 4 were heterofermentative *L. fermenti.* For these organisms the apparent cysteine requirement appears to be a need for a reducing substance and not for cysteine *per se.* Many *L. fermenti* strains evidently do not require cysteine though this may not always be apparent with some of the commonly-used synthetic media. 2. In contrast to *L. fermenti,* other lactobacilli—most *L. casei,* lactose-negative *L. casei, L. acidophilus,* and the majority of *L. plantarum*—require cysteine for growth in the presence of ascorbic acid. The rate of growth of these lactobacilli often is accelerated by ascorbic acid in the presence of either suboptimal or optimal amounts of cysteine. 3. Some implications of these findings concerning microbiological assay of cysteine and the distinguishing characteristics of heterofermentative *L. fermenti* and homofermentative lactobacilli are discussed.

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Comparative Study of Orosomucoid Preparations from Sera of Six Species of Mammals.* (22064)

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The presence of an electrophoretically and ultracentrifugally homogeneous acidic protein of very high carbohydrate content has been demonstrated in normal human plasma(1-4). This glycoprotein, designated orosomucoid (5,6), is one of the major components(5) of the "mucoprotein fraction" of human serum (6,7). "Mucoprotein" fractions have been demonstrated in the serum of many animal species(8-16). It appeared of some interest to determine whether proteins with characteristics of orosomucoid are also present in the blood of other species. In the present studies orosomucoid has been prepared from normal, pooled beef, guinea pig, horse, rabbit, and rat serum, and has been compared with that isolated from human plasma.

Materials and methods. Serum orosomucoid was separated by a procedure previously reported(1). To 500 ml of pooled serum from normal animals was added 500 ml of 0.1 M sodium acetate and 360 g of ammonium sulfate.† The precipitated proteins were

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* Supported by grants from the American Trudeau Society; American Cancer Society; and National Cancer Institute, National Institutes of Health, Public Health Service.

† Erroneously reported previously(1) as 2.73 Molar.

removed by filtration after 16 hours at 4°C. The pH was reduced first to 4.9 and then to 3.7, the precipitated proteins again being removed by filtration after 16 hours at 4°C following each adjustment. Precipitation of the orosomucoid preparations was achieved by saturation of the filtrate with ammonium sulfate, the precipitate then being dialyzed and lyophilized. Since the animal sera were usually hemolyzed, it was necessary to refractionate 1% solutions of the orosomucoid preparations at least once to obtain a white product. Due to the loss of material during refractionation, an accurate estimate could not be made of the amount of orosomucoid in the sera of the different species. The isolated proteins were readily soluble in distilled water, in solutions of neutral salts, and in dilute or concentrated solutions of acids and alkalis. Acidified solutions were not coagulated by boiling.

Methods. The hexose content of the preparations was determined by the orcinol method of Sörenson and Haugaard(17), using a 1:1 galactose-mannose standard. Hexosamine was determined by the Rimington(13) modification of the Elson-Morgan method. "Sialic acid" was determined by the direct Ehrlich reaction as described by Werner and Odin(18), using "sialic acid" isolated from submaxillary mucin as a standard.‡ Nitrogen was determined by digestion and direct nesslerization(19). Protein was determined by the biuret method of Mehl(20), employing bovine serum albumin as a standard. All values are expressed on a moisture-free basis and were obtained by drying each preparation to constant weight at 105°C. Electrophoresis was carried out with a modified Tiselius apparatus, or with an Antweiler micro-electrophoresis apparatus using a veronal-sodium chloride buffer at pH 8.5, and an acetate-sodium chloride buffer at pH 4.5, ionic strength 0.1, as previously described(1).

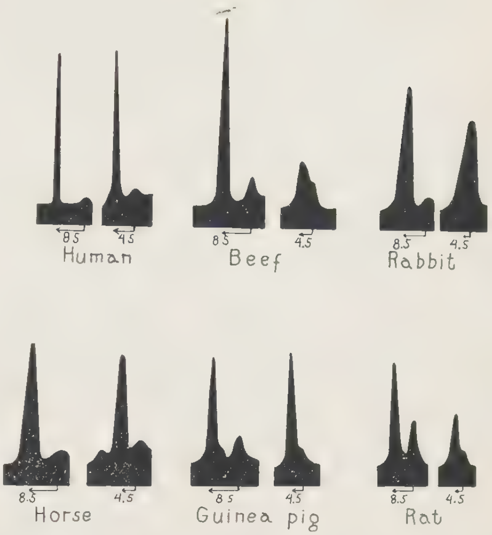

FIG. 1. Electrophoresis of orosomucoid preparations at pH 8.5 and 4.5. Ascending boundaries. Preparations from human, beef, guinea pig and rat with modified Tiselius apparatus. Rabbit and horse preparations with Antweiler micro-electrophoresis apparatus.

TABLE I. Electrophoretic Mobilities of Orosomucoid at pH 8.6 and 4.5.

	Mobility × 10^{-5} cm²/volt/sec	
Species	pH 8.5	pH 4.5
Human	−7.2*	−4.7*
Beef	−6.2*	−3.2*
Rabbit	−5.2†	−2.4†
Horse	−5.4†	−1.9†
Guinea pig	−5.8*	− .8*
Rat	−6.2*	− .8*

* Ascending boundaries with Tiselius apparatus.
† " " " Antweiler " .

Results. The results of the electrophoretic studies are shown in Table I and Fig. 1. Although electrophoretic homogeneity cannot be claimed for any of the preparations from animal sera, it is clear from the results at pH 4.5 that each preparation had an acidic protein as the major component.

Marked differences were apparent in the electrophoretic mobilities of the major component of different species, these differences being especially pronounced at pH 4.5. The fraction from human plasma had the greatest negative mobility at this pH (−4.7 x 10^{-5} cm²/v/sec), while the proteins from rat and from guinea pig serum had the lowest mobility at pH 4.5 (−0.8 x 10^{-5} cm²/v/sec). The major component of all preparations, however, was negatively charged at pH 4.5 and resembled, in this respect, the orosomucoid isolated from human plasma.

The results of the chemical analyses are

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‡ We wish to thank Doctors I. Werner, L. Odin and G. Blix for samples of "sialic acid."

TABLE II. Partial Chemical Composition of Orosomucoid Preparations.*

Species	Hexose	Hexosamine	"Sialic acid"	Nitrogen	Protein
	%				
Human	16.5	12.0	11.2	10.1	63.0
Beef	13.9	7.9	10.9	11.9	77.0
Rabbit	12.3	9.1	6.9	12.0	72.0
Horse	12.3	7.0	6.8	12.5	73.0
Guinea pig	11.1	6.6	6.2	13.2	84.0
Rat	9.9	6.4	5.1	13.6	88.0

* Moisture-free basis.

summarized in Table II. The orosomucoid preparations of each species were characterized by high hexose, hexosamine, and "sialic acid," and by low nitrogen and protein contents relative to the major components of serum.

The current study has demonstrated that the blood of several species of animals contains a glycoprotein analogous to the orosomucoid of human plasma. The most pronounced features of this similarity are the high hexose, hemosamine, and "sialic acid" contents; the stability to heat; and the acidic nature of all the preparations. While the similarities in the fractions from various species are marked, it is apparent that there are notable differences among them. The carbohydrate contents, the ratios of hexose to hexosamine or "sialic acid" and of protein to carbohydrate show considerable variation from species to species. Although the electrophoretic data are far too limited to permit definite conclusions, it seems probable that there are significant species differences in the isoelectric points of orosomucoid, the I.E.P. of the rat and the guinea pig preparations apparently being higher than that of human orosomucoid.

Summary. Highly soluble, acidic, carbohydrate-rich proteins have been isolated from the serum of 6 animal species by a fractionation procedure employing ammonium sulfate. All of the preparations contained large amounts of hexose, hexosamine, and "sialic acid," and were not coagulated by heat. The major component of each preparation had an isoelectric point below pH 4.5, and resembled the orosomucoid isolated from human serum. Differences in chemical composition and in electrophoretic mobility were observed in preparations from different species.

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Enzymatic Porphyrin Synthesis in Harderian Glands and Other Organs and Tissues of Mice.* (22065)

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The fact that the Harderian gland of mice of certain strains(1-3), rats(4), and hamsters (5) contain high concentrations of porphyrin is well established. Some investigators assumed that the red fluorescent gland was a secretory or porphyrin-synthesizing structure (6,1,4). On the basis of injection experiments(3,5), one of us stated that the Harderian gland appears to be an excretory structure and that its red fluorescence thus reflects the level of porphyrin metabolism of other tissues. The object of the experiments reported in this paper was to compare the porphyrin-synthesizing potentialities of the Harderian gland with those of other tissues and organs. It was hoped that this would definitely determine whether the Harderian gland is a secretory or an excretory structure.

The testing of the porphyrin-synthesizing potentialities of Harderian gland was made possible by the recent discoveries that certain cellular enzymes have the ability to synthesize porphobilinogen, uro, copro, and proto porphyrins from δ aminolevulinic acid(7,8). In these experiments, measured amounts of aqueous extracts of tissue homogenates were tested to determine their content of porphyrin synthesizing enzymes. It was found that the Harderian gland of mice contained a high concentration of porphyrin-producing enzyme as compared with other tissues, and it thus appears to be a porphyrin-synthesizing organ.

Materials and methods. The tissues to be tested were taken from 12 male mice of the Strong A strain. The mice were raised and housed in glass cages containing wooden shavings. They were fed Purina Laboratory Chow and given water without restrictions. The mice were anesthetized with ether and the tissues dissected out in a cold room (6°C). The Harderian glands were removed as rapidly as possible with care being taken to exclude extraneous tissues. One hundred milligrams of Harderian gland tissue was weighed out and placed in a mortar with a small amount of coarse grinding sand. To this was added 2 cc of 50:50 buffer-saline solution. The mixture was homogenized, rinsed from the mortar with 3 cc of the buffer-saline solution, and centrifuged at 2500 r.p.m. for 15 minutes. The supernatant material was used as the enzyme solution. The same procedure was followed to prepare enzyme solutions of brain, liver, and the femur with its attached muscles. To each of the 24 Klett tubes (6 for each tissue to be tested) 1.4 cc of 0.01 M δ aminolevulinic acid buffer-saline solution was added to act as the substrate. The buffer-saline solution consisted of equal volumes of isotonic sodium chloride solution and buffer solution - pH 7.2. The amounts of enzyme solution from each tissue to be tested were pipetted into the 6 tubes as follows: Tube No. 1-none (control); Tube No. 2 - 0.1 cc; Tube No. 3 - 0.2 cc; Tube No. 4 - 0.4 cc; Tube No. 5 - 0.8 cc and Tube No. 6 - 1.6 cc. Buffered-saline was added to each tube to bring the volume to 3 cc. The tubes were then stoppered with cellophane-covered corks and incubated at 37°C for 24 hours. At the end of this period, 6.5 cc of concentrated HCl was added to each tube to stop the enzymatic reaction and to clear the solution of any precipitate. This produced a 25% HCl solution. The tubes were then allowed to stand overnight in a cold room (6°C). A solution of hematoporphyrin hydrochloride in 25% HCl was prepared as a standard to be used in a Klett-Summerson filter photometer to determine the amounts of porphyrin formed. These experiments were repeated 4 times.

Results. The variation in the amount of porphyrin produced by different tissue enzyme preparations is shown in Table I. From this table, it may be seen that the amount

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TABLE I. Porphyrin Production by Aqueous Extracts of Harderian Gland and Other Mouse Tissue Homogenates. 12 mice in each series.

	μ of porphyrin/10 cc of reaction mixture/24 hr*				
	Enzyme concentration				
Tissues	.1 cc	.2 cc	.4 cc	.8 cc	1.6 cc
Harderian gland	7.1 ± .6	12.8 ± .4	32.3 ± .6	49.5 ± .9	100.7 ± 1.5
Liver	5.5 ± .3	9.5 ± .9	28.5 ± 1.6	34.60 ± .6	73.4 ± 1.8
Muscle and bone	1.8 ± .01	3.4 ± .2	6.7 ± .3	8.7 ± .2	16.1 ± .4
Brain	.8 ± .01	2.5 ± .2	4.4 ± .3	5.2 ± .4	9.7 ± .4

* Mean ± S.E.

of porphyrin produced in the tube containing the extract of the Harderian gland homogenate was much greater than in any other tissue tested. This was first noted when the tubes with the various enzyme concentrations were examined in ultraviolet light.

Other tissues tested (liver, muscle-bone, and brain) also showed a definite ability to produce porphyrin. The amount of porphyrin produced by the enzyme preparation from these tissues was not as high as that found for the Harderian gland. The porphyrin-producing potentialities of liver tissue was less than that of Harderian gland but 5 and 7 times as great as muscle-bone and brain respectively.

Discussion. The fact that the Harderian gland homogenate enzyme preparation produces a greater amount of porphyrin than the other tissues examined indicates that the Harderian gland is a porphyrin-secreting structure; at least the enzymes for manufacture of porphyrin from simple compounds are abundant in it. Porphyrinogenic enzymes are even more concentrated in the Harderian gland than they are in the liver or kidney. Gibson, Neuberger, and Scott(9) had reported that the liver and kidney of rabbits and the erythrocytes of birds contained considerable quantities of δ aminoleuvulinic acid dehydrogenase. Since the liver is one of the primary organs involved in porphyrin and heme degradation, the presence of large amounts of porphyrin-synthesizing enzymes in this organ seems paradoxical.

The much lower quantities of porphyrin-producing enzyme in the muscle-bone marrow homogenates and in the brain may be related to the low percentage of cellular material involved in the homogenate. Also, our results may have been influenced to a slight degree by the presence of variable amounts of blood in the tissues, as no attempt was made to wash the blood out before the tissues were homogenized. However, this could not explain the great variation which we observed. Analysis of these results showed, as might be expected, that the differences observed are highly significant ($P<0.01$).

Summary and conclusions. 1. Buffered saline extracts of equal weights of mouse Harderian gland, liver, muscle-bone, and brain homogenates were prepared. 2. Various amounts of these extracts were tested for their capacity to produce porphyrin by adding 1.4 cc of a 0.01 M solution of δ aminolevulinic acid and incubating the mixture in a stoppered Klett tube at 37°C for 24 hours. 3. The Harderian gland displayed the greatest porphyrin-producing enzyme activity, whereas the liver was less than that of Harderian gland but 5 and 7 times as great as muscle-bone and brain respectively. 4. These results led to the conclusion that the Harderian gland is a porphyrin-secreting structure and probably not an excretory type of gland.

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Effect of Heparin on Triglyceride and Free Fatty Acid Concentrations in Lymph.* (22066)

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The discovery by Hahn(1) that intravenously injected heparin abolished alimentary lipemia in dogs, is now well known. Clearing of lipemic serum did not occur *in vitro* upon addition of heparin itself(2,3); however, clearing of lipemic serum could be brought about by incubation with post-heparin plasma. Gofman *et al.*(4) in this laboratory have shown that plasma lipids are closely associated with a "spectrum" of lipoprotein molecules, which are distinguishable in the ultracentrifuge by virtue of their different densities. The effect of heparin *in vivo* on these molecules was found to be a conversion of the species of high flotation rates to those of lower flotation rates(5). Shore, Nichols and Freeman(6,7) in this laboratory also showed that clearing of a lipemic substrate by post-heparin plasma *in vitro* was accompanied by the release of free fatty acids. Robinson and French also reported the release of fatty acid when lymph was incubated with post-heparin plasma(8). Grossman *et al.*(9) have subsequently found that free fatty acid concentration in the plasma was increased after the injection of heparin, especially in the presence of lipemia. Similar observations have been made in both animals and humans in this laboratory.

It appears from the foregoing that the free fatty acids produced during *in vitro* or *in vivo* clearing of lipemia are at least partly the result of glyceryl ester hydrolysis, activated in some way by heparin. It is of interest to know whether such glyceryl ester hydrolysis can be demonstrated in the lymphatic system as well as in the blood. In the present communication evidence for lipolysis of triglycerides with release of fatty acids in the lymphatic system is presented.

A. *Materials.* 1. Animals: Male New Zealand white rabbits of body weight between 3 and 3½ kg and male Long Evans rats, 250-350 g were used. All animals were fasted for 12 to 18 hours before cannulation, unless otherwise stated. 2. Anesthetics: Nembutal, 50 to 60 mg/kg body weight administered intravenously for rabbits; 30 to 40 mg/kg for rats intraperitoneally. Ether was used to supplement nembutal anesthesia. 3. Heparin, aqueous solution, 10000 units/ml was used intravenously in doses of 15 mg/kg for rabbits and 5 mg/kg for rats. 4. C. P. ether, methanol, chloroform and carbon disulfide were used (J. T. Baker Chem. Co.). Commercial grade hexane was redistilled. 5. Trielaidin was prepared in this laboratory by reaction of elaidoyl chloride and glycerol. Infrared spectrum of partially purified product used for feeding indicated that small amounts of partial glycerides and free fatty acid were present. These do not interfere with the experiment, since presumably they would be formed in the digestive process.

B. *Methods.* 1. Collection of lymph: a) Cannulation of rat lymphatics—method of collection is essentially the same as that employed by Bollman *et al.*(10). b) Cannulation of rabbit lymphatics: In different experiments lymph was collected from 3 different regions, thoracic duct, hepatic lymph vessel and intestinal lymphatic vessel. A section of polyethylene tubing was used as cannula. For collection of thoracic duct lymph, tubing was cannulated into thoracic duct at junction of left subclavian and left internal jugular veins. For collection of hepatic lymph tubing was inserted into hepatic lymphatic duct at point approximately 1 cm caudal to liver near hepatic artery. Intestinal lymph was collected from main intestinal lymphatic vessel, along coeliac artery, just above left adrenal gland. Cannulae were left in place post-operatively and lymph in each case was collected continuously 48 hours after rabbits had recovered from anesthesia. During each study lymph

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* This work was supported in part by the U. S. Atomic Energy Commission and by the Lederle Laboratories Division of American Cyanamid Co.

TABLE I. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Thoracic Lymph of 5 Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml)						Free fatty acids (mg/100 ml)					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean
−2.0	102	—	104	—	—	103	11	—	10	—	—	11
.0	100	106	101	114	113	106	13	14	12	10	8	12
.5	58	59	81	88	72	72	52	61	31	36	21	38
1.0	36	45	32	42	32	37	21	29	29	30	38	29
2.0	28	33	21	32	40	31	18	21	20	20	18	19
2.5	40	39	25	22	55	36	16	20	20	19	21	20
3.5	50	—	—	25	78	51	20	—	—	19	26	22
4.5	—	93	—	—	—	93	—	18	—	—	—	18
6.0	—	—	94	—	—	94	—	—	17	—	—	17
12.0	—	—	—	103	—	103	—	—	—	18	—	18
24.0	—	100	—	—	—	100	—	16	—	—	—	16

samples were collected at 30 minute intervals. 2. Lipids of lymph were extracted and separated by method essentially similar to that of Borgstrom(11,12). Extracted lipids were separated into 3 fractions by elution from silicic acid columns. Triglycerides and free fatty acids were determined quantitatively by infrared spectrophotometry(7). For determination of elaidic acid, free fatty acids were separated from glycerides by extraction from petroleum ether solution with dilute alkali in 50% methanol(11). Content of elaidic acid was measured in both glycerides and free fatty acids by infrared spectrophotometric measurements of absorption band at 10.3 μ. Basis of this method has been given by Shreve *et al.*(13). Since amounts of material and percentages of elaidic acid were both small in our experiments, we used a base line method of calculation instead of equations developed by those authors. Results by the two procedures agree well when total sample is large enough. For smaller samples, in particular free fatty acids of pre-heparin lymph, only maximum values have been quoted. As a result of fatty acid exchange in processes of digestion and absorption, glycerides subsequently found in lymph are presumed to contain partially elaidinated fat molecules. The analysis gives equivalent amount of trielaidin.

Results. 1. Thoracic Duct Lymph (Rabbit and Rat). All lymph samples collected from the thoracic duct of rabbits before administration of heparin, 15 mg/kg intravenously, were visibly turbid. Post-heparin samples were clear. Table I summarizes changes in triglycerides and free fatty acids in thoracic duct lymph of 5 rabbits. Concentration of triglyceride decreased about 35% within first half hour after administration of heparin. The decrease reached its maximum, an average of 74%, in 2 hours. After that time its concentration increased gradually to the original level. Free fatty acids, on the other hand, increased by about 360% in the first 30 minutes. The increase in free fatty acids after administration of heparin does not compensate for the decrease in triglycerides. The conditions of the *in vivo* experiment are not such that an accurate balance of triglyceride and fatty acid changes can be expected, and also incomplete hydrolysis of triglyceride probably occurred. Similar results obtained in rats are shown in Table II. Smaller doses of heparin were effective in this species, about 5 mg/kg being comparable with 15 mg/kg in the rabbit.

TABLE II. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Thoracic Lymph of 5 Rats.

Time after heparin (hr)	Triglycerides (mg/100 ml)						Free fatty acids (mg/100 ml)					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean
0	207	218	214	220	239	220	25	39	28	35	48	35
1	116	112	160	128	221	147	140	90	73	57	160	104
2	105	125	135	105	224	138	160	192	56	101	213	144
3	—	—	—	—	127	127	—	—	—	—	128	128

TABLE III. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Intestinal Lymph of Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml)				Free fatty acids (mg/100 ml)			
	1	2	3	Mean	1	2	3	Mean
0	316	289	308	304	19	8	17	15
1	287	290	292	289	24	10	16	17
2	150	200	187	179	25	18	18	21
3	61	148	123	111	16	19	30	22
4	34	41	80	52	19	23	30	23

The average maximal drop (47%) in triglyceride concentration was at 3 hours post-heparin. Maximal increase in free fatty acid concentration (290%) was observed in 2 hours.

2. Intestinal lymph (Rabbit). The fat content of intestinal lymph was much higher than that of thoracic and hepatic lymph. The results of heparin injections are presented in Table III. It is notable that elevation of free fatty acid concentration is much less than for thoracic duct lymph. Also the lowering of triglycerides proceeds to a greater extent and for a longer period of time. 3. Hepatic lymph of normal rabbits was watery clear. The fat content of hepatic lymph was lower than that of lymph collected from either thoracic duct or intestinal lymph. Sixty minutes after administration of heparin, there was a slight change in triglycerides and a relatively large percentage increase in free fatty acids. Table IV shows triglyceride and free fatty acid contents of hepatic lymph before and after administration of heparin.

4. Trielaidin Feeding. To learn more about the origin of free fatty acids produced in lymph after heparin administration, tracer experiments were performed using trielaidin as a labeled fat. Rats cannulated in the thoracic duct as described were fed (by stomach tube) a suspension of 50-100 mg trielaidin in 2 ml of non-fat milk. Heparin was given 3-3½ hours after this feeding, and lymph samples were collected and analyzed. Results are summarized in Table V.

TABLE IV. Effect of Heparin on Concentrations of Triglycerides and Free Fatty Acids in Hepatic Lymph of Rabbits.

Time after heparin (hr)	Triglycerides (mg/100 ml)				Free fatty acids (mg/100 ml)			
	1	2	3	Mean	1	2	3	Mean
0	65	70	60	65	7	10	7	8
1	65	45	25	45	70	31	32	44
2	45	33	20	33	76	54	46	58
3	29	—	16	23	40	—	43	42
4	51	—	18	35	39	—	40	39
5	56	—	—	56	41	—	—	41

5. *In vitro* effects. If lipolysis occurs in post-heparin lymph *in vivo* it may be inferred that the active factor is present in such lymph and should be demonstrable by *in vitro* test. Such confirmation is provided by two observations. (1) Pre- and post-heparin rabbit hepatic lymph were incubated with a fatty substrate prepared from rat lymph by centrifuging 3½ hours at 20000 r.p.m. In each case 0.2 ml of top 1 ml from this centrifugation was mixed with 1 ml of lymph to be tested for lipolytic activity and 0.5 ml of saline, and the mixture incubated 3 hours at 38°C. Glycerides and free fatty acids were determined in the same way as described for the *in vivo* experiments. (2) Analyses were made of fresh pre- and post-heparin rabbit thoracic duct lymph and compared with results obtained on same samples after they remained at 4°C for 3 weeks. The results for these two experiments are presented in Table VI.

Discussion. The data that have been presented demonstrate an effect in the lymph of both rabbits and rats, following intravenous injection of heparin, that is evidently parallel to the effect in blood. The triglyceride content drops, and the amount of free fatty acids increases. It is further indicated that in the absence of excess fat influx from the intestine (*i.e.*, in fasted animals) a predominant share of free fatty acids found in thoracic duct lymph has arrived there via the hepatic lymph channel. This suggests an implication of the liver as principal source of fatty acids under these conditions. However, if trielaidin is fed and heparin is given during absorptive state, 10-20% of free fatty acids formed consist of elaidic acid. Thus it appears that hydrolysis of some newly absorbed fat has taken place. This hypothesis is further supported by ability of post-heparin lymph to produce lipolysis *in vitro*. However, an alternative explanation which has not been ruled out is that absorp-

TABLE V. Changes in Elaidin and Free Elaidic Acid in Rat Lymph after Heparin. (Concentrations are in mg/100 ml.)

Exp.	Time with respect to heparin inj.	Total glyceride	Elaidin (equivalent trielaidin)	Total free fatty acids	Free elaidic acid
1	–2	299	26	—	—
	0	540	75	51	<5
	1	314	40	192	12
2	–3	236	51	37	<5
	0	304	62	61	6
	2	137	40	189	20
3	–3	189	32	57	<8
	0	207	47	29	<8
	2	116	35	156	16
4	–3½	264	59	30	<8
	0	570	175	50	<8
	2	365	143	136	26

tion process from the intestine may have been so altered that a larger fraction of fatty acids is passed into lymph without being reconstituted as glycerides.

Summary. The glyceride and free fatty acid contents of thoracic, intestinal, and hepatic lymph of rabbits, and thoracic lymph of rats were determined before and after intravenous administration of heparin. The principal effects observed were the following: 1. The turbidity that was always observed initially in thoracic or intestinal lymph was diminished or disappeared. Hepatic lymph was not originally turbid. 2. In all cases the glyceride content was reduced and free fatty acids increased. 3. Relative amount of glycerides was highest in intestinal lymph, and the change in that component was markedly greater than in hepatic lymph. 4. Hepatic lymph had a low initial content of glycerides, and showed a relatively larger change in free fatty acids. 5. Trielaidin was fed to rats and heparin given intravenously during absorptive phase. Elaidic acid was detected in free fatty acids subsequently appearing in lymph. Post-heparin rabbit lymph was capable of producing lipolysis of a fatty substrate *in vitro*.

TABLE VI. Lipolytic Activity of Post-Heparin Lymph *In Vitro*. (Concentrations are in mg/100 ml.)

	Glycerides		Free fatty acids	
	Initial conc.	Final conc.	Initial conc.	Final conc.
Exp. 1				
Pre-heparin	260	258	39	40
Post-heparin	233	196	75	115
Exp. 2				
Pre-heparin	116	112	10	14
Post-heparin	75	43	25	57

Exp. 1. Incubation of rat lymph lipids with rabbit hepatic lymph for 3 hr at 38°C.

Exp. 2. Rabbit thoracic duct lymph kept at 4°C for 3 wk.

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Daily Variations in Mitotic Activity of Adrenal Cortex, Thyroid and Oral Epithelium of the Rat.* (22067)

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Daily physiologic periodicity seems to characterize mitotic activity in the oral epithelium(1,2) as well as in a variety of other tissues from plants and animals (for review see 3 & 4). These daily changes in mitotic activity may be influenced by daily periodicity in various environmental factors which affect the organism. But intrinsic, partly hormonal mechanisms also may contribute to the 24-hour spacing of peaks in mitotic activity(2,4). In this latter connection, the following questions were explored in this study. First, whether day-night differences may be detected in the mitotic activity of certain endocrines under the standardized environmental circumstances and at those times of day at which significant day-night differences have been demonstrated in the oral epithelium(2). If day-night differences existed in the mitotic activity of the glands studied, it also was of interest to know whether the times of "high" and "low" in number of mitoses were roughly the same in endocrine and non-endocrine tissues.

Materials and methods. For 3 weeks prior to their sacrifice, 32 Sherman albino rats weighing about 135 g were kept in single cages in a room illuminated from 6:00 to 18:00, darkened from 18:00 to 6:00, and maintained at 24 ± 1°C and 49 ± 2% relative humidity. A commercial diet (Lab Blocks, Allied Mills, Chicago) and tap water were available to the rats *ad libitum* since weaning and throughout the period of standardization. A subgroup composed of 16 rats was decapitated between 7:00 and 7:30 ("Day-rats") and another subgroup of 16 rats between 21:30 and 22:00 ("Night-rats"). The adrenal glands, the thyroid and part of the oral epithelium were immediately fixed by means of Zenker-Formol. Serial sections, 4 μ thick, were then prepared and stained with Hematoxylin-Eosin. Mitotic activity in every section from each tissue and rat was then evaluated. In the case of the adrenal cortex, mitotic activity was related to area-units of 10 mm^2. For the thyroid, mitoses were counted in 150 oil-immersion fields(83 ± 1 cells per field). In the oral epithelium, mitoses were counted in units of 10 mm length of basal membrane. The length of basal membrane was measured by the use of a special device which will be described elsewhere(5).

Results. A summary of results in Fig. 1 shows the considerable variability of the data for individual rats, which are given by the height of the columns graphed. More important, some considerable day-night differences in mitotic activity also become apparent from a comparison of the horizontal lines, which denote the means in mitotic activity by day and by night. For the thyroid and for the oral epithelium, there is a day "high" and a night "low" in number of mitoses. By contrast, a night "high" and a day "low" appear to characterize mitotic activity in the adrenal cortex of the nocturnally active rat.

Discussion. These data seem to demonstrate, first, a 24-hour periodicity of mitosis in the thyroid and adrenal of the rat. Second, the data also reveal that daily mitotic periodicity in the thyroid is synchronized with the 24-hour spacing of mitosis in the oral epithelium. Mitotic periodicity in the oral epithelium, in turn, has been shown to be synchronized with 24-hour periodicity of mitosis in other tissues(2) and with the 24-hour rhythms in eosinophil count and in rectal temperature (1,2). It also has been shown that the adrenals are essential for the maintenance of 24-hour rhythms in eosinophil count(4,6) and in some aspects of hepatic phospholipid metabolism(7). If, as seems likely(4), the

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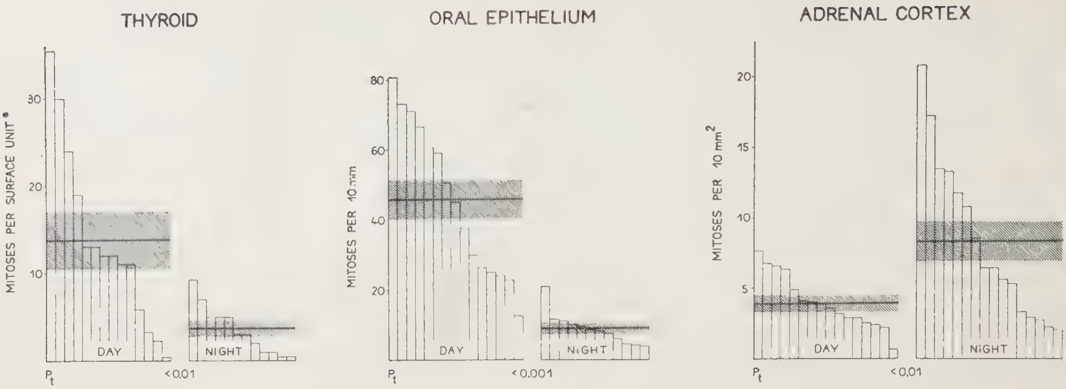

* Mitoses/150 oil-immersion fields.

FIG. 1. Mitotic activity at two times of day in tissues investigated. (Columns show data for individual rats; horizontal lines and shaded areas denote means ± 1 S.E.; day = 7:00-7:30; night = 21:00-21:30.)

adrenal constitutes a fundamental mechanism of physiologic daily periodicity, the phase-difference noted herein between its mitotic periodicity and the 24-hour spacing of other rhythms may call for a detailed analysis of adrenal mitotic activity (with more frequent sampling during the 24-hour period).

Summary. Daily periodicity characterizes mitotic activity in the adrenal, the thyroid and the oral epithelium of male Sherman albino rats, kept under standardized environmental circumstances. The daily times of "high" and "low" in number of mitoses are roughly synchronized for the thyroid and the oral epithelium, while the 24-hour periodicity in mitotic activity of the adrenal cortex shows a different timing.

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Oxytocic Action of Rat Hypothalamus Extracts. (22068)

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Vogt(1) confirmed the presence of vasopressor, antidiuretic and oxytocic activities in extracts of dog hypothalamus. According to her results the hypothalamus differs from the posterior pituitary in that the relative oxytocic activity of the hypothalamus is much lower than the expected value, the P/O ratio (pressor activity/oxytocic activity) being 14.6. Inasmuch as Lawler and du Vigneaud (2) have shown that oxytocin-free vasopressin possesses an oxytocic activity corresponding to 5% of the pressor activity, Vogt(1) suggests that the oxytocic substance extracted from the hypothalamus may be pure vasopressin. This interpretation is of importance in view of the findings of Bargmann and Scharrer(3) on production of hormones of the pars nervosa of the hypophysis by the neurosecretory nuclei of the hypothalamus. These observations led Vogt(1) to deduce that either the oxytocin is chemically changed by the hypothalamus or this organ produces oxytocin and its nuclei the vasopressin.

Methods. The comparative effect of several crystalline proteolytic enzymes: pepsin, chymotrypsin, trypsin and carboxypeptidase, on extracts of hypothalamus and posterior pituitary of the same rat was studied in order to determine whether the oxytocic activity of the hypothalamus might be due to a substance similar to either oxytocin or vasopressin. Croxatto(4) has shown that the oxytocic activity is destroyed by chymotrypsin, but it is not affected by carboxypeptidase, pepsin and trypsin; on the other hand vasopressin is inactivated by chymotrypsin and also by crystalline trypsin. Lawler and du Vigneaud (2) using a highly purified preparation of vasopressin have confirmed the latter finding. Extracts were obtained from the anterior hypothalamic region of male and female adult rats, comprising a triangular area based on the posterior boundary of the optic tractus, from which portions of the tuber cinereum were definitely excluded. The small portion tested contained the supraopticus and paraventricularis nuclei. The tissue thus obtained from 2 or 3 rats was ground with micronized quartz in a mortar, the temperature maintained at 0°C. The ground tissue was suspended in 0.9% NaCl, in the proportion of one ml per hypothalamus. The clear supernatant fluid obtained on centrifuging at low temperature was divided in 2 equal portions, to one of which 2 to 3 mg of enzyme were added. Both samples were incubated at 37°C for one to 6 hours at pH 7.3, except that in the case of pepsin the pH was 2.5. After incubation the extract was neutralized and tested on rat uterus. Some of the incubation mixtures were brought to pH 2.5 and deproteinized by addition of 10 volumes of alcohol, centrifuged, the alcohol in the supernatant evaporated and the residue taken up in Ringer's solution. The posterior lobes of the hypophysis of the same animals were subjected to the same treatment as the hypothalamus.

TABLE I. Oxytocic Activity (mU) Anterior Hypothalamus Extracts.

Before incubation	After incubation			Hr
	No enzyme	Enzyme added		
.75	.5	.7	Pepsin	1
	.5	.5		1
	.5	.75		5
1.-	1.0	1.0		5
.5	.5	.5		5
	1.5	.5	Chymotrypsin	1
	*1.5	1.5		1
	1.0	.5		1
	1.0	.1		2
	1.0	.1		2
.5	.4	0		6
	.2	0		6
	.5	.75	Trypsin	4
.5	.2	.2		6
	.2	.2		6
	.40	.5		6
	.2	.4		6
.5	.3	.3		6
.6	.5	.5	Carboxypept.	2
	.3	.3		4
	† .2	.4		4
	.3	.3		6

Extracts were incubated at pH 7.3 except in the case of pepsin the pH was 2.5; in (*) the pH was 3 and in (†) the pH was 8.2.

Results. The effect of pepsin, carboxypeptidase, trypsin and chymotrypsin on extracts of hypothalamus was the same as that observed for extracts of the posterior pituitary (Table I). Chymotrypsin was the only enzyme that caused a complete or almost complete loss of the oxytocic activity of hypothalamus and posterior pituitary extracts. No decrease in oxytocic activity of both extracts was produced by the other enzymes tested. In some experiments trypsin, carboxypeptidase and pepsin produced a slight increase of the oxytocic activity of both extracts. Incubation of the extracts at pH 7.3 with no addition of enzyme resulted in an appreciable loss of oxytocic activity by both extracts.

If one takes into consideration the specificity of the enzymes tested, our results would strongly support the hypothesis of the chemical identity of the substances from the hypothalamus and the posterior pituitary of rats. Contrary to the assumption of Vogt(1) regarding dog hypothalamus, the oxytocic activity of rat hypothalamus appears to correspond to that of a substance like oxytocin rather than one like vasopressin.

Summary. A study of the effect of pepsin, carboxypeptidase, trypsin and chymotrypsin on the oxytocic activity of extracts of the rat hypothalamus and posterior lobe of the hypophysis. Both extracts show similar behavior. Chymotrypsin is the only enzyme that produces complete or almost complete loss of the oxytocic activity. No decrease in oxytocic activity is produced by the other enzymes. Incubation of the extracts at pH 7.3 without enzymes results in an appreciable loss of oxytocic activity by both extracts.

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Antithyroid Properties of 2-Amino, 5-Nitrothiazole in Rats.* (22069)

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The report that 2-amino, 5-nitrothiazole caused the complete suppression of sexual manifestations and functions in fowl(1-3) has been explained as being the result of inhibition of secretion of pituitary gonadotropin(3). Although the effect of the compound appeared to be restricted to fowl(3), interest in a possible antigonadotropic substance that is not related to the sex-hormones has prompted the present investigation of 2-amino, 5-nitrothiazole utilizing the rat as the experimental animal.

Materials and methods. Eighteen litters of Long-Evans female rats from 45 to 49 days of age were assigned to 4 groups so that each group contained 18 rats from different litters and so that each group had the same average body weight. At this time 2 groups were ovariectomized and 2 groups were sham-operated. One operated group and one sham-operated group was started the next day on commercial 2-amino, 5-nitrothiazole[†] added to the diet at a concentration of 0.3%. All other rats received the same diet (commercial laboratory chow[‡] that is adequate in iodine) with no drug added. All rats were maintained on their respective diets and tap water *ad libitum* until sacrifice 30 days later. The intact animals were given a 2 μc tracer dose of carrier free I^{131}[§] by the intraperitoneal route and sacrificed 24 hours later. The operated animals were handled exactly as above except that they were sacrificed 48 hours post I^{131} administration. At sacrifice the body, pituitary, thyroid, adrenal and uterus weights were recorded and the thyroid glands were placed in 2 cc of Bouin's fluid and assayed for radioactivity in a well-type scintillation counter against 3 aliquots of the original I^{131} as prepared for injection. The radioactivity measurements were expressed as the percentage of injected dose found in the sample and the counting statistics were such that the resulting probable error was less than 1%. The thyroid glands were then sectioned and stained for histological study. In another experiment 30 day-old male Sprague-Dawley rats were assigned to 3 groups of 15 animals so that the average body weight of all groups was the same. These rats were maintained

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[†] "Enheptin", a commercial product containing aminonitrothiazole not less than 20%, soybean oil meal, not more than 80%, crude protein not less than 33%, crude fat not less than 0.3% and crude fiber not more than 7%, sold by American Cyanamid Co. (Lederle Laboratories Division), New York City.

[‡] Purina Laboratory Chow. Ralston Purina Co., St. Louis, Mo.

[§] Obtained from Oak Ridge National Laboratory.

TABLE I. Effect of Feeding 2-Amino, 5-Nitrothiazole (0.3% of Diet Adequate in Iodine for 30 Days) to Intact and Ovariectomized Female Long-Evans Rats on the Body Weight, Adrenal Weight, Thyroid Weight, Pituitary Weight, Uterus Weight and Percent of Administered I^{131} Accumulated by the Thyroid Gland (24 Hr between Isotope Administration for Intact Animals and 48 Hr for Ovariectomized Animals). Mean ± stand. error reported.

Treatment	No. of animals	Body wt (g)	Adrenal (mg)	Thyroid (mg)	Pituitary (mg)	Uterus (mg)	I^{131} (%)
Intact	18	154 ± 4	40.3 ± 2.2	9.2 ± .5	6.20 ± .24	181 ± 23	6.35 ± .48
Intact + 2-amino, 5-nitrothiazole	18	142* ± 4	31.8† ± 1.7	11.1† ± .4	5.96 ± .24	141 ± 17	3.12† ± .27
Ovariectomized	17	161 ± 4	38.6 ± 2.0	9.6 ± .5	7.35 ± .20	40 ± 3	4.39 ± .30
Ovariectomized + 2-amino, 5-nitrothiazole	16	144* ± 4	31.3† ± 1.3	11.4† ± .5	6.84 ± .26	35 ± 2	.60† ± .11

* P_{05} when tested by t-test against mean immediately above.
† P_{01} " " " " " " " " .

TABLE II. Effect of Feeding 2-Amino, 5-Nitrothiazole (0.30%) or Purified 2-Amino, 5-Nitrothiazole (0.06%) in a Diet Low in Iodine to Male Sprague-Dawley Rats for 8 Days on Body Weight, Testis Weight, Thyroid Weight, Pituitary Weight, and Percent of Administered I^{131} Accumulated by the Thyroid Gland (18 Hr between Isotope Administration and Sacrifice). 15 animals in each series.

Treatment	Body wt (g)	Testis (g)	Thyroid (mg)	Pituitary (mg)	I^{131} (%)
.06% 2-amino, 5-nitrothiazole	91 ± 1	1.21 ± .04	7.5 ± .3	3.86 ± .12	17.1 ± 1.6
Low iodine	92 ± 2	1.22 ± .32	6.6* ± .2	3.84 ± .22	25.4† ± 1.6
.30% 2-amino, 5-nitrothiazole	92 ± 2	1.16 ± .18	8.1† ± .4	3.82 ± .10	16.7† ± .9

* † Same as in Table I.

on a diet low in iodine‖ for 8 days(4). One group received commercial 2-amino, 5-nitrothiazole added to the diet at a concentration of 0.30%, another group received the purified compound¶ at a concentration of 0.06%, while one group received no drug treatment. All animals were maintained on these diets and tap water *ad libitum* until sacrifice. Eighteen hours before sacrifice each rat received a tracer of I^{131} and radioactivity of the thyroid gland determined as before.

Results. The feeding of a commercial preparation of 2-amino, 5-nitrothiazole (0.30% of a diet adequate in iodine) for 30 days to intact or ovariectomized Long-Evans rats had no effect on pituitary, or uterus weights. The 2-amino, 5-nitrothiazole treated rats had smaller body and adrenal weights, larger thyroid weights, and lower 24 hour and 48 hour thyroidal accumulation I^{131} values than non-treated rats (Table I).

Male Sprague-Dawley rats fed either a commercial preparation of 2-amino, 5-nitrothiazole or a purified preparation of the compound (0.30% or 0.06% respectively of a diet poor in iodine) for 8 days had no effect on body, testis or pituitary weights. However, the 2-amino, 5-nitrothiazole treated rats had larger thyroid glands and a lower 18-hour thyroidal accumulation of I^{131} than the non-treated rats (Table II).

In all groups treated with 2-amino, 5-nitrothiazole the thyroid glands gave a histological picture of columnar follicular cells and less densely staining colloid that is consistent with effects given by treatment with antithyroid compounds of the thiouracil type (Fig. 1, 2).

Discussion. 2-amino, 5-nitrothiazole had no effect on gonad function, as judged by testis weights or uterus weight, when administered to male or female rats. This is in agreement with previous reports(3) since the effectiveness of 2-amino, 5-nitrothiazole in suppressing gonad function was limited to fowl and could not be extended to mammals.

‖ Remington Diet No. 348. Each 100 g contains: wheat gluten, 16 g; dried yeast, 4 g; corn meal 78 g; $CaCO_3$, 1 g; and NaCl, 1 g.

¶ 2-amino, 5-nitrothiazole, Lot No. 251T2F, assay 97.2% pure, contributed by Lederle Laboratories.

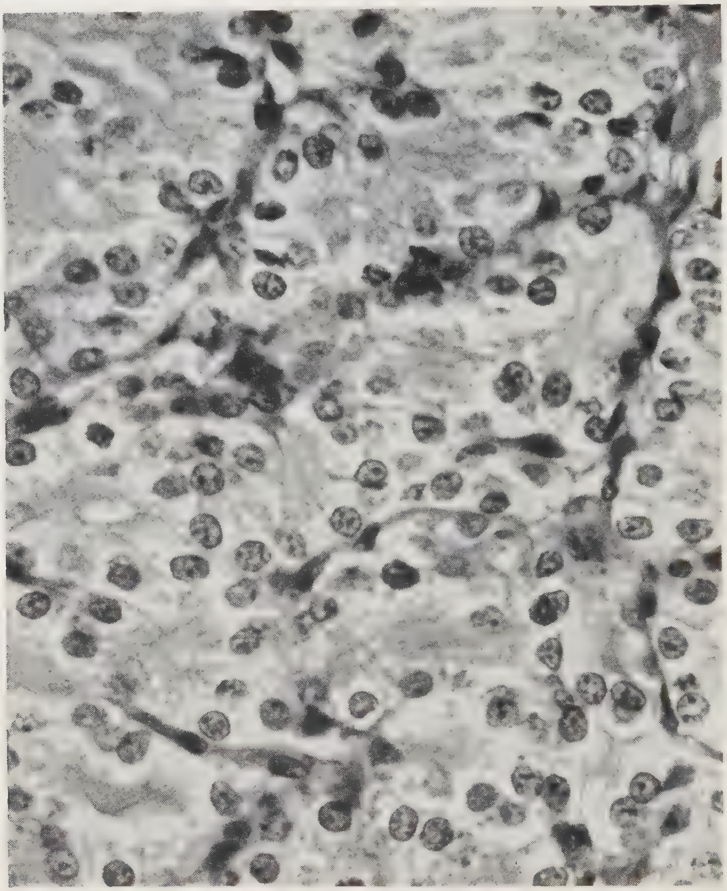

FIG. 1. Thyroid gland from female Long-Evans rat that had been fed 0.30% 2-amino, 5-nitrothiazole for 30 days. Bouin fixation, hematoxylin and eosin. (×600.)

However, there seems to be little doubt that 2-amino, 5-nitrothiazole had a definite antithyroid action in these rats since the thyroid weight and follicular cell height was increased and the thyroidal accumulation of I^{131} was lowered in rats treated with the compound. It is not surprising that 2-amino, 5-nitrothiazole possesses antithyroid properties since it is well known, in man at least, that aminothiazoles possess definite antithyroid properties that produce an increase in thyroid weight and a lowering of I^{131} uptake. In fact, the aminothiazoles have been used for the control of hyperthyroidism(5). It seems likely that the other changes noted in the aminonitrothiazole-treated rats, *i.e.,* a depressed body and adrenal weight, might also be ascribed to an induced hypothyroidism since these changes are not inconsistent with changes produced by hypothyroidism(6). Indeed, it is possible that the suppression of sexual function in fowl noted following treatment with the compound might also, at least in part, be ascribed to a relative lack of thyroid hormone since various degrees of hypothyroidism may contribute to a lack or regression of gonad function and the anterior pituitary responses to changes in gonad function(7). The fact that the gonadal suppression noted in 2-amino, 5-nitrothiazole treated fowl has not been attributed to a decreased thyroid function does not detract from the foregoing suggestion because the only tests reported on thyroid function in fowl treated with 2-amino, 5-nitrothiazole were tests designed to show if the compound suppresses the pituitary production of thyrotropin and were not designed to test the possibility of a direct action of 2-

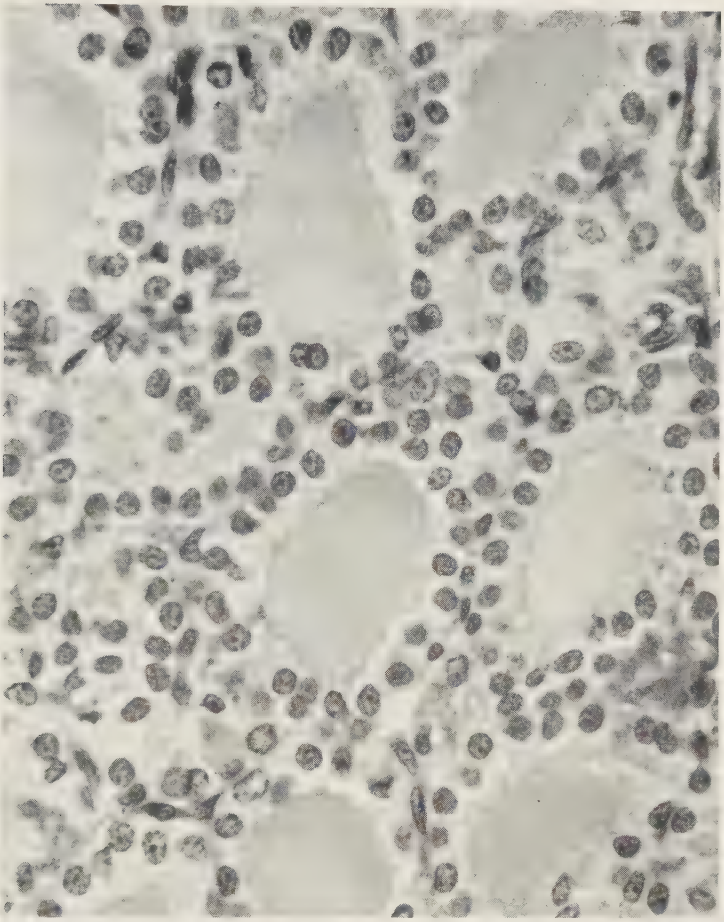

FIG. 2. Thyroid gland from control female Long-Evans rat of same age as in Fig. 1. Bouin fixation, hematoxylin and eosin. (×600.)

amino, 5-nitrothiazole on the thyroid gland or its production of thyroid hormone. Also, the reports that this compound interrupts egg laying(8,9) are consistent with the suggestion that 2-amino, 5-nitrothiazole might produce abnormalities in pituitary-thyroid function by reason of its antithyroid properties.

Summary. Rats fed 2-amino, 5-nitrothiazole had larger thyroid glands and a lower thyroidal accumulation of I^{131} than nontreated rats. It is suggested that the antithyroid properties of this compound might explain the inhibition of secretion of pituitary gonadotropin that has been reported following the administration of 2-amino, 5-nitrothiazole to fowl.

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Leukopenic and Thrombocytopenic Effect of Hypothermia in Dogs. (22070)

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(Introduced by G. M. Guest.)
(With the technical assistance of Marilyn McG. Helmsworth.)
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Serious cardiac arrhythmias and cessation of respiratory center activity are two major complications of hypothermia which have caught the attention of investigators studying the effects of cooling. The purpose of this report is to describe another complication observed in a high proportion of our experiments. Leukopenia and a thrombocytopenia often developed in dogs which were subjected to rapid cooling; this marked reduction in the number of thrombocytes may be related to the hemorrhagic phenomena which were also encountered in many of the animals. During early investigations of hypothermia in this laboratory we noted that a large proportion of the dogs bled from the gastro-intestinal tract during the first 48 hours after rewarming(1). This loss of blood was a complication seen more frequently in the dogs subjected to experiments in hypothermia than in a similar group of animals subjected to major operative procedures with normal body temperature. In the course of another group of hypothermia experiments a second manifestation of this hemorrhagic diathesis was noted. Dogs that were cooled to the range of 18° to 26°C were subjected to exploratory thoracotomy. In most of these animals the surfaces of the wounds oozed blood in a way not observed in the course of operations upon animals with unimpaired coagulation mechanisms. This faulty coagulation was no doubt the cause of the unusually high incidence of hemothorax in the animals which survived the experiment. Several earlier reports mentioned intestinal tract bleeding as a complication in dogs that had been cooled(2,3). While this complication was manifest in more than one-half of the animals cooled in our laboratory, the impression gained from publications is that others have not observed this type of bleeding so frequently. The mechanism of the diathesis has not been explained, although Ross has noted prolongation of the clotting time in his experiments with cooling by an extracorporeal technic(4).

There is also a clinical aspect to this problem. Even though it cannot be stated in absolute terms at this time, it appears that impairment of the coagulation mechanism may develop in patients subjected to hypothermia as currently employed for cardiovascular surgery(5). It seemed advisable, therefore, to plan a group of experiments in rapid cooling with special attention to the effects upon the blood cellular elements, as the first step in an attempt to elucidate the problem of post-hypothermia bleeding in dogs.

Method. The mongrel dogs used were 8 months old or older; those with infection or malnutrition were excluded from the group for these experiments. Excitation of the animals during the administration of the anesthetic was avoided as far as possible. Pentobarbital sodium was administered intravenously in average dose of 250 mg. The trachea was intubated at once. In the more active animals this was facilitated by the administration of small amounts of ether by open drop. Cyclopropane and oxygen were administered thereafter, and this proved to be an ideal anesthetic in all experiments in which hypothermia was induced. However, the animals with normal body temperature in the control series required additional doses of diphenhydramine hydrochloride (Demerol) to maintain anesthesia at a satisfactory plane. All of the dogs breathed actively during the period of observation and no mechanical ventilator was employed. Animals were supported in the prone position, tilted so that the extended hind legs were at a level 6 inches higher than the head. The dorsal surface of the thorax was shaved and cracked ice was packed about all of the body except the head and neck. Brine was circulated through this loose ice pack to speed the cooling. Additional procedures in the experiments involved only minor surgery. A ligature was passed around the trachea and tied so that no gas escaped around the in-lying tracheal catheter. The venous pressure was measured through a catheter inserted into the superior vena cava. Samples of venous blood drawn after the animal was anesthetized were obtained through the same catheter; the blood was allowed to drip into heparin tubes and syringes were not employed. The blood pressure was measured with a mercury manometer, attached to a cannula in the femoral artery. Temperature changes were measured by a thermometer inserted into the rectum. Samples of venous blood were kept chilled in a pan of cracked ice. The counts of erythrocytes, platelets(6) and leukocytes(7), as well as the preparation of blood smears and determination of hematocrit(8), were completed within 4 hours after the samples were collected.

Results. Control experiments were carried out on 5 dogs. The standardized method for anesthesia was used for this group, but it did not prove as satisfactory as when it was employed in the hypothermia series (see Method). The conditions of the experiment did not vary in any other respect; the animals in the control group had their backs shaved and were subjected to the same procedures as those employed in the hypothermia group for cannulation of the trachea and an artery and vein. Observations were continued over a period slightly longer in the series of control animals than in the group of 19 that were placed in cracked ice. The average period of observation was 157 minutes for the control group and 100 minutes for the group subjected to cooling.

Acidosis which was most likely respiratory in origin, was of comparable severity in both groups of animals. This fact was ascertained by the measurements of pH and carbon dioxide content in samples of arterial blood drawn at regular intervals during the course of the experiments. The average of the lowest rectal temperatures observed in the animals in the chilled group was 24.3°C.

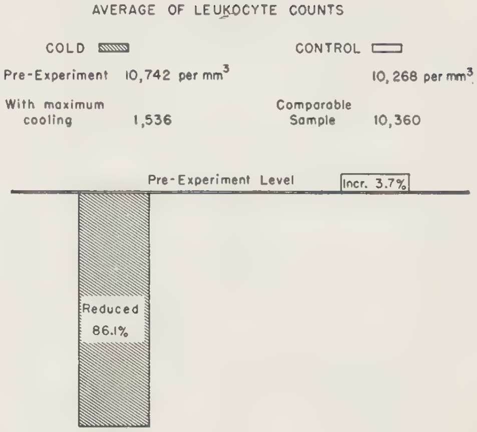

FIG. 1.

Changes in the hematocrit were similar to those that have been reported on many occasions by earlier investigators. The dogs that were cooled had some hemoconcentration with an average increase in hematocrit of 9 vol. %. The mean corpuscular hemoglobin concentration fell somewhat with the average change 1.7%. The mean corpuscular volume increased with the average rise 7.5(9) cubic micra.

There was a marked change in the total leukocyte count produced by hypothermia. Fig. 1 tabulates the averaged results in the cooled group for comparison with the averaged changes in the controls. An 86% reduction in leukocyte count was associated with the lowered body temperature. A differential count of cells was not made from every sample. However, the changes were consistent in all samples that were examined completely. The percentage of lymphocytes in the smears increased and the percentage of neutrophils fell to approximately one-third of the value obtained at normal temperature. The conditions of the control experiments resulted in a small increase (3.7%) in the leukocyte count. This change in the control series is not statistically significant.

The total eosinophil count was also altered in these experiments. In Fig. 2 the results in the hypothermic group are compared with the results from dogs at approximately normal

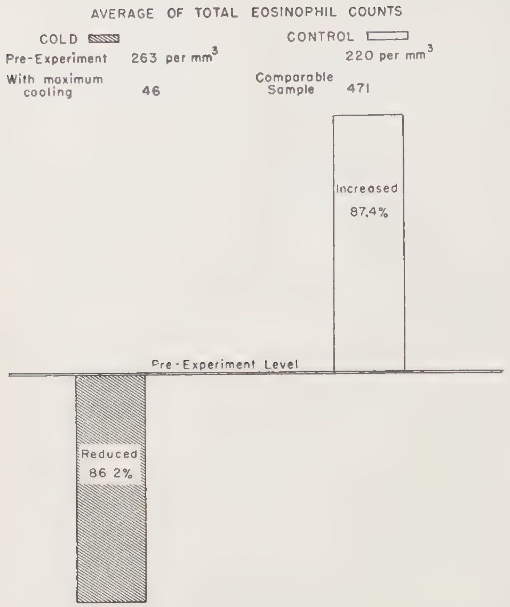

FIG. 2.

body temperature.* The average of the fall in total eosinophil count was 86.2% in the hypothermic group. In the controls, however, this count was increased 87.4%.

Another outstanding change observed in these experiments was in the platelet count (Fig. 3). The change observed in the control experiments was a reduction in the count which averaged 16.5%. The dogs that were cooled had an 81.7% reduction in the count of platelets. In several of the experiments no platelets could be found in the samples of blood drawn at the time of the maximum cooling effect. The samples of blood remained uncoagulated when allowed to stand for two hours in Lee-White tubes at room temperature.

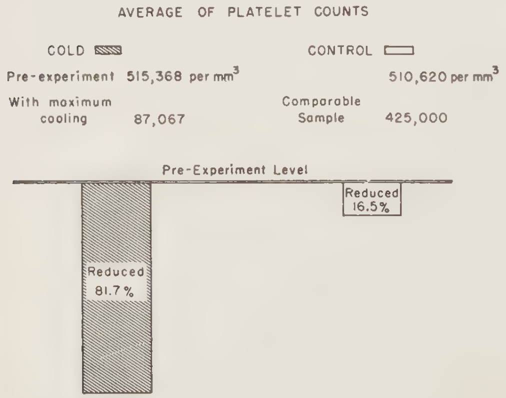

FIG. 3.

Summary. 1. Attention is drawn to the complication of impaired coagulation encountered in experimental hypothermia in dogs. 2. The method used for anesthetizing and rapidly reducing the body temperature by packing in cracked ice is described. 3. The following changes were associated with hypothermia: The leukocyte count was reduced an average of 86%, the total eosinophil count was reduced an average of 86%, and the platelet count was reduced an average of 81%. 4. The coagulation mechanism was not studied directly, but data from these experiments suggest that the strong thrombocytopenic effect observed may be a factor in the impaired coagulation.

The authors wish to acknowledge the assistance given by Miss Jocelyn Simpson and Miss Joan Hamme in preparation of the manuscript.

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* The range of temperatures was from 36° to 37°C. Slight cooling resulted from washing, shaving, and the anesthetized state.

In vitro Anaphylaxis in the Sensitized Mouse Uterus. (22071)

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Anaphylaxis in the mouse has been studied by many workers, and most of their findings indicate that histamine plays little or no role in anaphylactic shock in this species. Using the Schultz-Dale technic of *in vitro* anaphylaxis, this paper is concerned with an attempt to rule histamine in or out as being part of the basic mechanism in the reaction and to study the effect of pertussis vaccine on *in vitro* anaphylaxis.

Materials and methods. Schultz-Dale technic in mouse anaphylaxis. Fourteen virgin female Swiss mice over 3 months old were injected subcutaneously with 0.6 ml of alum precipitated egg albumin[†] prepared by the method of Proom(1). This amount contained 15 mg of the antigen. The mice were used 2 to 4 weeks later, at a time when, as judged by gaping of the vaginal orifice, they were in estrus, and the uterus consequently larger.[‡] After sacrificing the animal by stretching its neck, the uterus was immediately removed and placed in a modified Ringer's solution(2). It was kept at 0°-4°C until used, usually within 2 hours. Two aerated tissue baths, each containing 45 ml of the Ringer's solution maintained at 38°-39°C were set up. One-half of one uterine horn was suspended in one bath; one-half of the other horn in the other bath.[§] After 30 minutes, the lever on each of the strips was connected to a kymograph, and the pattern of normal contractions obtained. Crystalline egg albumin[‖] was then added to each bath to a concentration of 15 μg per ml of bath. All additions were made with a 3-inch needle attached to a tuberculin syringe, with the tip of the needle bent in a manner to insure spraying of the liquid. After contraction of the strip, which invariably occurred within 30 seconds after the first addition of antigen, the tissues were washed, the normal contractions recorded, and a second addition of the same amount of egg albumin made to test for desensitization. When desensitization had been effected, to test the reactivity of the tissue, acetylcholine[¶] was added to a concentration of 10 μg per ml, and the contraction recorded. At least one tissue strip from each of the 14 mice was found to react to egg albumin. In 13 of the mice, at least 2, and sometimes 3 or 4 reacted; in only one mouse was just one piece reactive. Duplicate contractions of tissues from the same uterus were usually but not always of a similar magnitude. The occasional dissimilarity in reactivity of different portions of the uterus is in line with similar observations on the guinea pig ileum(3). A typical contraction is shown in Fig. 1.

Tissues from 12 of the mice did not react on the second addition of antigen; but those from the other 2 mice were not desensitized until 3 additions of antigen had been made. Similar observations have been reported for the guinea pig(4). After desensitization, all of the tissues reacted to acetylcholine in a concentration of 10 μg per ml of bath. The uteri from 10 normal mice were similarly tested. In no case was there a reaction to egg albumin; but in every case a reaction occurred in response to the 10 μg per ml of acetylcholine.

Role of histamine and acetylcholine in mouse anaphylaxis. This experiment was de-

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[*] This work supported by a Grant-in-Aid from the American Cancer Society upon recommendation of Committee on Growth of the National Research Council.

[†] Albumin, Egg Impalpable Powder, J. T. Baker Chem. Co.

[‡] After this first experiment was completed, it was found that administration of an estrogen 4 to 5 days prior to testing resulted in uniformly large uteri. In all other experiments to be reported mice were injected intramuscularly with 8.8 γ of *Progynon-B* (Schering Corp.) in mineral oil prior to testing.

[§] In some instances, the other 2 pieces were tested in a similar manner.

[‖] Armour and Co. preparation.

[¶] Acetylcholine chloride, Matheson Co.

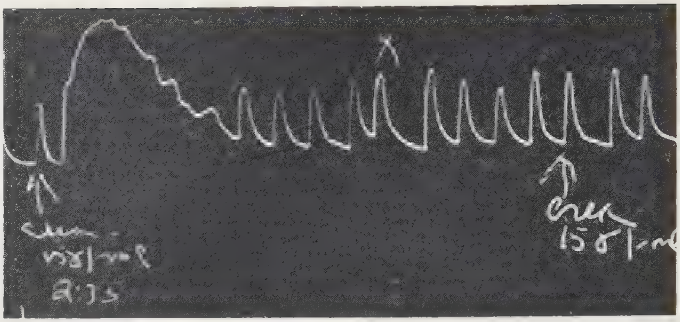

FIG. 1. Typical anaphylactic contraction of actively sensitized mouse uterus. Arrow = crystalline egg albumin added 15 μg/ml. × = contraction following wash.

signed to determine the role of histamine and acetylcholine by blocking reactions due to these drugs with Benadryl and atropine, respectively. It was first demonstrated that these drugs could block the reactions when histamine or acetylcholine were added to the sensitized mouse uterus; but that they could not block the reactions when antigen was added to the sensitized uterus. Ten mice actively sensitized as before and 3 mice passively sensitized by the intra-abdominal injection 4 hours prior to testing of 0.4 ml of pooled serum from actively sensitized mice were used in this study. This antiserum contained sufficient antibody for a positive precipitin reaction when antigen in a concentration of 0.01 mg per ml was added.

In preliminary studies it was found that histamine in a concentration of 50 μg of histamine** base per ml of bath was necessary to produce a uniform uterine contraction of the sensitized uterus. Benadryl†† in a concentration of 0.4 to 0.8 μg per ml of bath was always sufficient to prevent the reaction to histamine. Strips not responding to histamine in the presence of Benadryl would, after washing, respond to a corresponding dose of histamine.

It was also shown that atropine‡‡ in a concentration of 2 μg per ml of bath was invariably sufficient to prevent the reaction to 10 μg or more of acetylcholine per ml of bath.

The technic of testing was as described previously. It was first established that a positive reaction to egg albumin could be obtained on one-half of one uterine horn. Then 2 fresh pieces of the same uterus were set up in the 2 water baths. To one of these Benadryl was added to a concentration of usually 0.4 μg per ml of bath; to the other, atropine was added to a concentration of 2 μg per ml of bath. After about one minute, the antigen was added as before. In some instances, the fourth piece of tissue was tested in the presence of both atropine and Benadryl.

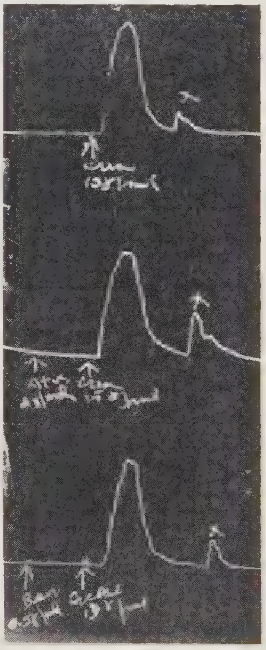

FIG. 2. Persistence of anaphylactic contraction in presence of atropine and Benadryl. × = contraction following wash.

** Crystalline histamine diphosphate, Eimer and Amend, dissolved in phosphate buffered saline, pH 7.2.

†† Parke, Davis and Co. preparation.

‡‡ Obtained from local pharmacy.

In all 13 mice—10 actively and 3 passively sensitized—neither Benadryl nor atropine inhibited or diminished the anaphylactic contraction (Fig. 2).

Uterine mast cells. In view of reports indicating that histamine may be released from tissue mast cells(5,6), an experiment was done to determine whether there were any changes in the uterine mast cells subsequent to *in vitro* anaphylaxis. Two strips of uterus from an actively sensitized mouse were placed in Ringer's solution as usual. After shocking a strip *in vitro,* it was placed in 10% formalin; the second, non-shocked strip served as a control. The tissues were prepared by a standard histological technic. In the 3 times the experiment was done, the mast cells were abundant and easily identified, but there were never any discernible changes in their morphology after shock.

TABLE I. Effect of Pertussis Vaccine on Anaphylaxis and Histamine Sensitivity *in Vitro.**

Exp.	Normal	Ea	Ea + .1 pertussis 4-5 days earlier	Ea + .2 pertussis 4-5 days earlier	Ea and pertussis injected together
A+† H+		8/12‡	3/12	2/8	3/9
A+ H−		1/12	2/12	2/8	0/9
A− H+		2/12	6/12	3/8	3/9
A− H−		1/12	1/12	1/8	3/9
Total H+	3/18	10/12	9/12	5/8	6/9
Total A+		8/12	5/12	4/8	3/9

* All mice received estrogen intramuscularly 4-5 days prior to testing.

† A = anaphylaxis; H = histamine—50 γ/ml of bath.

‡ No. positive/No. tested.

Effect of pertussis vaccine on anaphylactic and histamine sensitivity in vitro. It has been shown that an injection of pertussis vaccine increases both histamine and anaphylactic sensitivity in the intact mouse. This experiment was done to determine whether similar findings could be observed when the tests were done *in vitro.* Five groups of female Swiss mice over 3 months old were used in this experiment. The first group of 18 were not injected, and served as controls. Mice in the other 4 groups were all injected subcutaneously with the 0.6 ml of alum precipitated egg white as before. One of these 4 groups received no further treatment. The other 3 groups received pertussis vaccine as follows: 9 mice were injected intra-abdominally with 0.1 ml of pertussis vaccine§§ immediately after the egg white injection; a group of 12 received 0.1 ml of pertussis vaccine 4 to 5 days prior to testing for sensitivity; and the last group of 8 received 0.2 ml of pertussis vaccine 4 to 5 days prior to testing. On the 10th, 11th, 12th, and 13th day following the egg white injection, proportionate numbers from each of the 5 groups were tested for anaphylactic sensitivity as previously described until all had been tested. They were tested for histamine sensitivity by recording the reaction to 50 μg of histamine base per ml of bath.

In these, and in all following experiments, only one strip was tested if the reaction were positive; 2 strips if negative. The results of these experiments are recorded in Table I.

The results of the experiment on the effect of pertussis vaccine on anaphylactic and on histamine sensitivity are expressed in Table I. To determine any difference in sensitivity to histamine and to anaphylaxis, the groups were compared statistically using the method of χ^2. Each group was compared with every other group in the 2 categories.

Analysis of the data reveals that pertussis vaccine had no effect on anaphylactic sensitivity as tested *in vitro* when given 4 days prior to testing at 2 dosage levels; or when administered with the sensitizing dose of antigen. Nor does pertussis vaccine have any more effect on histamine sensitivity as measured *in vitro* than does the injection of the sensitizing antigen alone. None of the treated groups were statistically different from each other, but all were statistically different from the normals.

Discussion. Numerous publications have questioned the role of histamine in mouse anaphylaxis. The relative insensitivity of the intact mouse to histamine(7-9), the suggestion that the body of the mouse does not contain sufficient histamine to produce a reaction (7), the observation that antihistaminic drugs have little if any effect on anaphylaxis (10), and the inability to establish a correlation between histamine and anaphylactic sensitivity in this species(10,11), all indirectly suggest that histamine is not concerned in anaphylaxis in the mouse.

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§§ Pertussis vaccine, Cutter lot No. E5452; 0.1 ml contains 6 x 10^9 organisms.

By using the Schultz-Dale technic of *in vitro* anaphylaxis, it has been shown in this study that the isolated tissue, like the intact animal, is very resistant to histamine; and that the sensitized mouse uterus reacts to the specific antigen to the same extent in the presence of a demonstrably anti-histaminic drug as it does in the absence of that drug. Furthermore, no change in the tissue mast cells, believed to be involved in the release of histamine(5,6), was manifested after the *in vitro* anaphylactic reaction. These findings strengthen the hypothesis that histamine is not concerned in mouse anaphylaxis.

The reaction also proceeds to its full extent in the presence of atropine, a finding which would argue against an important role for acetylcholine.

The fact that pertussis vaccine increases both histamine and anaphylactic sensitivity in the intact mouse has been amply confirmed (8,9,12). Studies on the isolated tissues, however, reveal that anaphylactic sensitivity to the egg white antigen was not increased; and that the increase in the histamine sensitivity was of the same order if the animal received the antigen alone, or the antigen plus pertussis organisms. In light of the finding that the tissues were all similarly sensitive, this suggests that the effect of pertussis vaccine in increasing anaphylactic sensitivity in the intact animal must be through alteration of some mechanism other than an immunological one. The finding that the egg white injection increases histamine sensitivity to the same extent that pertussis vaccine does (Table I) is difficult to explain. Many bacterial antigens have been tested(10,13) to determine whether or not they increased sensitivity to histamine, but only those prepared from *Brucella abortus* were found to have any effect. Parventjev(14), however, has shown that injection of a mouse with Sarcoma 180 tends to increase the histamine sensitivity.

Summary. 1. The Schultz-Dale technic for demonstrating anaphylaxis *in vitro* was used successfully on uteri from egg white sensitized mice. 2. The anaphylactic reaction persisted in the presence of either atropine or Benadryl, thus ruling out any major role for histamine or acetylcholine in mouse anaphylaxis. 3. The uterine mast cells were found to be morphologically unaffected by *in vitro* anaphylaxis. 4. Pertussis vaccine administered under varying conditions did not increase *in vitro* sensitivity to anaphylaxis. Immunization with egg white alone increased histamine sensitivity *in vitro* as much as did the pertussis vaccine.

We are indebted to D. L. Smith for advice on pharmacological procedures; and to C. D. Fink for statistical appraisal of the data.

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Precipitin Analysis of C-Reactive Protein by Gel Diffusion.* (22072)

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C-reactive protein (C-RP), an abnormal protein present in the "acute phase" serum, has been under investigation for the past several years. This substance, which forms a precipitate with the non-type specific C-polysaccharide of the pneumococcus in the presence of Ca^{++}(1), was found by electrophoresis, to migrate between the α_2 and β globulin fractions(2). The C-reactive protein was separated from the human serum(3) and was prepared in crystalline form from human serous fluids(2). A number of investigations have shown the presence of C-RP to be a very sensitive indicator of activity in rheumatic fever(4-6). Evidence has been presented that C-RP may be present in cancer(7) and in acute myocardial infarction(8). In rheumatoid arthritis and active tuberculosis, the C-RP is significantly correlated with seromucoid(9). Small amounts of this abnormal protein may be detected in human serum by a precipitin test utilizing specific antiserum from rabbits hyperimmunized with purified C-RP.

The present study was carried out during the course of studies on the biological properties of C-reactive proteins. With the use of agar gel diffusion technics it was possible to obtain quantitative and qualitative values of C-RP in the serum of seventy-one patients with a variety of illnesses.

Procedure. C-RP antiserum—Three New Zealand Red rabbits (2 kg) were injected intravenously, twice a week for 3 weeks, with 0.5 cc of a solution containing 1 mg/cc of crystalline C-RP. 1.5 cc of Freund's adjuvant(10) was incorporated with 1 mg of crystalline C-RP and injected subcutaneously into 2 separate abdominal sites at the time of the first intravenous injection. On the fourth week the rabbits were bled and the sera were preserved with merthiolate in a final concentration of 1:10,000. The *crystalline C-RP* was prepared from 2 liters of fluid obtained by thoracocentesis from a patient with Hodgkin's disease. The isolation and crystallization of C-RP was performed following the method described by Wood *et al.*(11). The total yield of crystallized material was 28 mg. *Human sera* were obtained from the serology laboratory at the Mount Sinai Hospital, Chicago, from 71 patients with a wide variety of illnesses. The age of the patients ranged from 12 to 74 years. *The quantitative precipitin technic* for the determination of antibodies in rabbit C-RP antisera was that described by Kabat and Mayer(12). The amount of antigen was added in slight excess to produce a total antibody precipitation and the total nitrogen precipitated was determined by the microkjeldahl technic. Each analysis was carried out in duplicate. Tests of the supernatant fluid showed that the dilution of crystalline C-RP reacted with antibodies in the region of antigen excess. The amount of antibody nitrogen precipitated at the equivalence zone from each rabbit antiserum was, respectively 0.652 mg, 0.615 mg, and 0.485 mg per cc of antiserum. *Agar precipitin* (*Oudin*) *technic*—The modification of Munoz and Becker(13) was employed. The serum agar contained 1 part of rabbit antiserum diluted 1:5, 1 part of 0.6% agar which had been previously adjusted to pH 7.5 and clarified in 0.15 M NaCl. After cooling, 0.4 ml of the antigenic solution was overlayed on the agar. Several dilutions of the C-RP solution in 0.15 M NaCl were used to determine the rate of penetration of the precipitating bands. The serial dilutions of this antigenic solution contained respectively 0.080, 0.040, 0.020, 0.010, 0.005 mg of crystalline C-RP per ml. *Agar precipitin* (*Ouchterlony*) *tech-*

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* This investigation was aided in part by a grant from the Asthmatic Children's Aid and Schieffelin and Co.

[†] Research Fellow, Allergy Research Unit, Chicago Medical School.

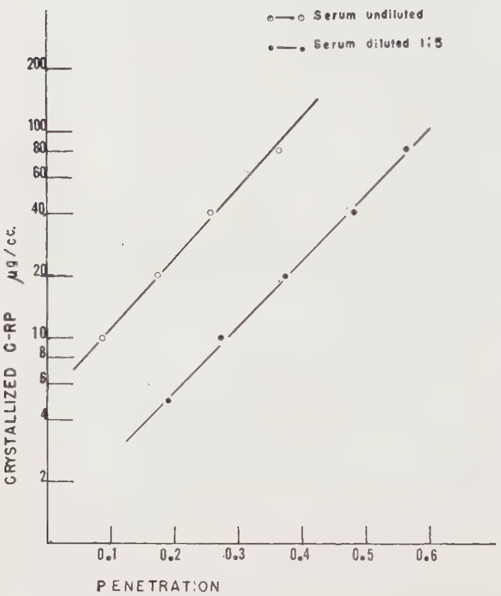

FIG. 1. Penetration values of serial dilutions of crystalline C-reactive protein into agar-serum. Serum contained 0.625 mg of antibody N/ml. Penetration values computed from formula: Penetration (P) = Distance (D)/$\sqrt{\text{Time (T)}}$.

nic—The Ouchterlony technic was used as modified by Halbert *et al.*(15). 0.15 ml of rabbit antiserum was placed in the central well, and 0.1 ml of patients sera in the peripheral wells. The plates were then placed in the refrigerator at 6°C and the precipitin bands read at intervals starting from 7 days. The final readings were done after 24 days.

Results. Crystalline C-RP obtained from the thoracocentesis fluid of a patient with Hodgkin's disease, showed precipitin bands migrating in antiserum-agar medium. The migration of the bands followed a straight line relationship with the square root of the time. The rate of penetration appeared to be directly proportional to the log of C-RP concentration and inversely proportional to the antibody content of the rabbit antiserum. Fig. 1 was obtained by plotting the penetration against the concentrations of crystalline C-RP (respectively, 0.08, 0.04, 0.02, 0.01, 0.005 mg/ml). The rabbit antiserum contained 0.652 mg of antibody nitrogen per cc and was used diluted 1:5 and undiluted.

With antisera containing a known concentration of antibodies, the penetration rates of the C-RP present in the blood of 16 patients with rheumatic fever were determined. The rates of penetration ranged between 0.652 and 0.100. The concentration of the protein was calculated by the use of the standard curve of penetration of the crystalline C-RP (Fig. 1), and was expressed in terms of mg of protein per cc of serum. The values ranged between 0.140 and 0.003 mg/ml.

We further studied the immunological reactions between C-RP and C-RP antiserum by the technic devised by Ouchterlony(16). When crystalline C-RP was allowed to diffuse against rabbit C-RP antiserum, 3 distinct bands appeared, one sharp and 2 faint. Increasing the concentration of the crystalline C-RP increased the distance between the precipitation bands and the antigen wells; but no variations in the shape of the lines of precipitation were noted. The sera of 71 pa-

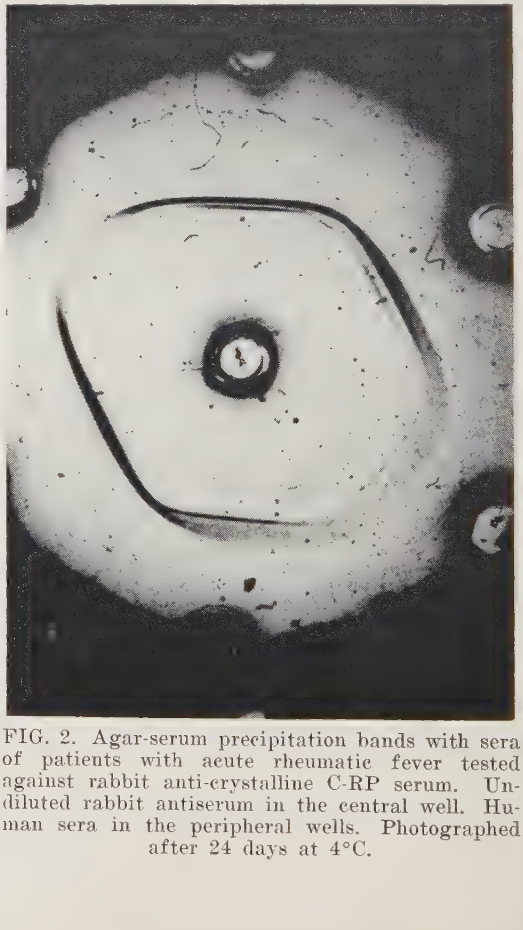

FIG. 2. Agar-serum precipitation bands with sera of patients with acute rheumatic fever tested against rabbit anti-crystalline C-RP serum. Undiluted rabbit antiserum in the central well. Human sera in the peripheral wells. Photographed after 24 days at 4°C.

tients with different diseases were studied with the Ouchterlony technic. From 0 to 3 bands of precipitation were formed in the agar medium. Sometimes it was difficult to define the exact number of the formed bands. However, more than 3 precipitation bands were never observed. When 2 or 3 lines were noted, one was normally quite intense and common to all the sera; others were moderately to very delicate. The sharpness of the bands also showed considerable differences. Some were clearly defined, others faint and diffuse. The heaviest common line was apparent first; the others took several more days to appear. The final configuration of the bands was present after 3 weeks (Fig. 2). No relationship was observed between the concentration of the C-RP in the blood (as determined by the penetration rate of the main band in the Oudin tubes) and the number and shape of the precipitation lines present in the agar plates.

Several controls confirmed that no cross reactions occurred between human normal sera and the rabbit C-RP antisera used. No bands appeared when sera containing high levels of C-RP were allowed to diffuse against pneumococcus C-polysaccharide.

Discussion. The total amount of C-RP that is present in the "acute phase serum" is very important in evaluating an inflammatory process. It is most helpful in those cases in which the concentration of the C-RP in the serum is low, and when other laboratory tests and clinical evidence of activity are equivocal (4,14). The use of the agar diffusion technic offers the possibility of evaluating with a high degree of accuracy very small amounts of the C-RP present in the serum.

Each precipitin band represents an antigen-antibody system(16-17), therefore, at least 3 antigens are present in the C-RP in its crystalline form. These 3 antigens are present in "acute phase sera" in different concentrations. The quantity of one antigen is not apparently related to the amount of the others. The possibility has been suggested by Jennings (18) that multiple bands represent several determinant groups of a single antigen. From the data reported here, both of these possibilities are likely.

The results obtained indicate that an inflammatory stimulus causes the appearance in the blood stream of varying amounts of C-RP which may give rise to differences in its antigenic components. Determination of the values of these components may be of importance in studying the progress of an inflammatory process.

Summary. 1. The rate of penetration of crystallized C-RP into agar-antiserum medium has been determined. 2. With this procedure, the C-reactive protein present in human serum of 16 patients with rheumatic fever was evaluated. Variations of less than 0.001 mg of C-RP per ml of serum were detectable. 3. By observing the pattern of the precipitin bands at least 3 antigenic components were found to be present in crystalline C-reactive protein. 4. In human serum, the C-RP present appeared to contain up to 3 antigenic components in different concentrations.

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Triazenes as Inhibitors of Mouse Sarcoma 180.* (22073)

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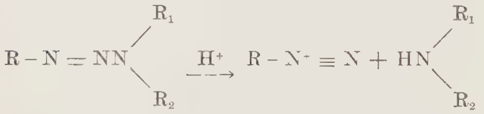
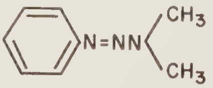
3,3-Dimethyl-1-phenyltriazene (Fig. 1) has recently been found to inhibit *in vivo* growth of the mouse sarcoma 180 (S-180). Tumor inhibitory activity has not previously been described for triazenes; thus a new biological activity is disclosed for derivatives containing the diazo moiety to which this activity might be ascribed. The triazenes are characterized by a high degree of lability in the presence of H-ions, resulting in the formation of a diazonium ion and an amine, thus:



$$R-N=NN\begin{matrix}R_1\\R_2\end{matrix} \xrightarrow{H^+} R-N^+\equiv N + HN\begin{matrix}R_1\\R_2\end{matrix}$$

The vigorous coupling activity of the diazonium ion is well known and this particular property of the triazenes ("diazoamines") has been utilized to advantage in the synthesis of azo dyes(1), for polymerization in the rubber industry(2), and for alkylation of dissimilar hydrocarbons in the synthesis of high-octane gasoline(3). It has heretofore been demonstrated that S-180 is sensitive to alkylating agents such as the mustard gases(4), triethylene melamine(5), and the polyfunctional phosphoramides(6). It therefore seemed reasonable to assume that a compound such as 3,3-dimethyl-1-phenyltriazene (hereafter to be referred to as "the triazene") could achieve systemic distribution intact and, at the cellular level, be subject to H-ion catalysis with liberation of benzenediazonium ion and dimethyl amine. The diazonium ion might then inhibit S-180 by reacting with elements essential for cell proliferation—a phenomenon postulated for other alkylating agents(7). This hypothesis will be examined below. It remains to be seen whether the triazenes are sufficiently selective for tumor cells over normal, rapidly proliferating tissue to be useful in higher mammals. In the mouse the therapeutic index is fairly low.



3,3-DIMETHYL-1-PHENYLTRIAZENE

FIG. 1.

Special emphasis will be given the triazene described above since it has proved so far to be the most interesting member of the series (Table I). A study of other diazoamines is continuing in an effort to establish a correlation between structure and tumor-inhibitory activity.

Materials and methods. Dimethyl-phenyl-triazene[†] was synthesized by one of us (C. S. R., Jr.)(8). The compound is a pale, amber fluid with a specific gravity of 1.02.

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[*] This investigation was supported in part by an institutional research grant from the American Cancer Society, by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of National Research Council and by a grant from the National Cancer Institute, U. S. Public Health Service.

TABLE I. Triazene Analogs Which Have Been Adequately Tested for S-180 Inhibition.

Compound	Source
Positive*	
3,3-Dimethyl-1-phenyltriazene	Dr. C. S. Rondestvedt, Jr. (U. Mich.)
3,3-Dimethyl-1-p-nitrophenyltriazene	"
3,3-Dimethyl-1-p-tolyltriazene	"
5(1-Naphthylazo) guanylurea	American Cyanamid Co.
1(5-Chloro-2-tolylazo)-5-(4-methoxy-2-sulfophenyl) biguanide	"
Negative	
3,3-Diethyl-1-phenyltriazene	Dr. R. K. Barclay (Sloan-Kettering)
Diazoaminobenzene	"
4,4'-(3,3'-Dimethoxy-4,4'-biphenylenebisazo)-morpholine	Eastman Kodak Co.
(3,3'-Dimethoxy-4,4'-biphenylene) bis-3,3-dimethyltriazene	"
1-[3,3-bis(2-Cyanoethyl)triazeno]-anthraquinone	"
1-(3,3-Dimethyltriazeno)-anthraquinone	"
1,1'-(4,4'-Biphenylene)bis(3,3-dimethyltriazene)	Parke, Davis & Co.
N^1-Phenylazo-N^2-phenylurea	Dr. Karl Pfister (Merck & Co.)
1-p-Nitrophenyl-3-phenyltetrazene	Remington Rand
1-p-Carboxyphenyl-3-phenyltetrazene	"
4-[3,3-bis(2-Hydroxyethyl)triazeno]benzenesulfonamide	Chem.-Bio. Coordination Center
1,3-di(4-Methoxyphenyl)triazene	Confidential
Diazo-1-amino-2-naphthol-4-sulfonic acid	National Aniline Co.
2,2'-Diazoaminofluorene	Dr. F. E. Ray (U. Florida)
4-(p-Nitrobenzenediazoamino)azobenzene	Purchased

* At tolerated doses tumor avg diameters of treated mice were 75% or less of the avg diameters of control tumors.

It is characterized by a pungent, aromatic odor and is irritating to mucous membrane when applied undiluted. Uniformity and stability of the various samples used were evaluated by ultraviolet spectrophotometry. The triazene is stable in alkali, but is rapidly decomposed by acid. It is, however, but sparingly soluble in water. For tumor inhibition studies, 18-22 g Swiss albino mice (Millerton Research Farms) were employed. Except where possible sex differences were to be explored, it was customary to use female mice. Single trocar implants of S-180 weighing *ca* 5 mg (wet weight) were made subcutaneously in the right axillary region. The implants were cut from tissue excised from donor mice after 7 days of growth. Usually therapy was initiated by direct oral intubation or by intraperitoneal injection 24 hours post-implantation and was continued for 7 successive days. Preliminary experiments established that it was immaterial whether the daily dose was given at one time or divided equally and the 2 half doses given at 7-hour intervals. Therefore the daily dose, uniformly contained in 0.1 ml of peanut oil/20 g mouse, was given as a single injection. Control mice received peanut oil alone by the appropriate route. All animals were weighed on the day of implantation and the day following the last injection. Tumors were measured through the skin with calipers. Two perpendicular diameters were determined and the mean obtained for the purpose of expressing tumor size as an "average diameter." In some experiments, the mice were sacrificed immediately thereafter and the tumors excised and weighed for the purpose of demonstrating the relationship between mass and average diameter.

Results and discussion. Intraperitoneal doses of the triazene employed in this study

TABLE II. 3,3-Dimethyl-1-phenyltriazene Inhibition of S-180. Treatment given intraperitoneally and continued for 7 days.*

Daily dose, mm^3/kg	Avg wt change, g	Avg tumor diameter, mm	Avg tumor wt (wet) mg
62.5	−3	7.0	89.5
31.25	0	7.9	153.6
15.63	+1	9.3	234.2
0.00	+2	10.8	389.5

* Composite data from 3 separate experiments; a total of 60 mice/dose level.

[†] Original sample was supplied through the Chemical-Biological Coordination Center, Washington, D. C., as also was the case with 3,3-dimethyl-1-p-nitrophenyltriazene.

TABLE III. 3,3-Dimethyl-1-phenyltriazene Inhibition of S-180 and Toxicity to Host following Oral Intubation. Therapy continued for 7 days.

Daily dose, mm^3/kg	Avg tumor diameter, mm	Toxicity	
		Avg wt change, g	Mortality
250	6.4*	−5.3*	16/20
125	6.4	−4.0	0/20
62.5	8.5	−2.0	”
0.0	12.0	−1.0	”

* Based on 4 surviving animals only.

have ranged between 62.5 and 15.63 cm/kg/day; with these 2 extremes and an intermediate dose of 31.25 cm, a 3-point curve may be constructed from wet weight data (Table II) which is a straight-line function when the *log* of the dose is plotted against per cent inhibition of tumor growth. Thus, a linear dose-response relationship exists between the inhibitor and its effects on S-180 growth with a 50% effective dose of approximately 22 cm; this was found to be the case, irrespective of sex of the host. Table II also shows the relationship between "average diameter" and the actual mass of tumor tissue. In the tumor-bearing Swiss mouse, repeated daily doses of the triazene greater than 62.5 cm/kg proved lethal to most animals prior to completion of a 7-day course (Table III). Indeed, the 62.5 cm regimen resulted in delayed lethality not attributable to tumor growth (Table IV).

The above tolerated dosage schedule of the triazene was also used to treat animals with "established" tumors. Intraperitoneal therapy was begun 96 hours after tumor implantation and was continued for 7 days thereafter. The relative growth retardation produced by each dose level was comparable with that seen when therapy was started at the earlier post-implantation interval.

Since the triazene is acid-labile it was anticipated the agent would prove ineffective when administered by the oral route unless precautions were taken to neutralize gastric juice. The triazene, however, not only produced tumor-growth inhibition but also host intoxication in the same order of magnitude of dose when administered either orally or by intraperitoneal injection (Table III). Other than dissolving in peanut oil, no precautions were taken to protect the compound from attack by gastric acidity.

S-180 grew, but failed to resume *rapid* growth, following an inhibitory course of the triazene. This observation suggested viability of tumor cells, or their proliferative capacity, had been seriously impaired by the agent. Gross examination of treated tumors revealed a firm, compact consistency with no evidence of extensive necrosis, suggesting that necrotizing activity by the agent was not responsible for sluggish growth. To explore this point further, a series of mice implanted with S-180 was treated by intraperitoneal injection for 7 days with 62.5 cm/kg/day of the triazene. Pieces from treated and control tumors were implanted into normal mice the day following the last injection. One week later the treated S-180 had attained an average diameter of 4.7 mm whereas untreated tumor had grown to an average diameter of 12.8 mm (20 mice/group). There were no instances of failure to "take" among the reimplanted, treated tumors which indicated that some, if perhaps not all, cells remained viable.

Alkylating agents such as nitrogen mustard and triethylene melamine (TEM) characteristically cause depletion of the cellular elements of the marrow in intoxicated animals (9). Since there existed a reasonable expec-

TABLE IV. Relationship between S-180 Inhibitory Doses and Delayed Lethality to Host of 3,3-Dimethyl-1-phenyltriazene. Seven successive days of intraperitoneal administration.

Daily dose, mm^3/kg	Tumor-bearing mice			Non-tumor-bearing mice	
	End of therapy		Mortality 7 days later	End of therapy mortality	Mortality 7 days later
	Avg tumor diam., mm	Mortality			
62.5	7.2	1/30	16/30	0/30	11/30
31.25	8.9	0/30	1/30	”	0/30
15.63	11.8	1/29	9/29	”	2/30
0	13.5	0/30	3/30	”	0/30

TABLE V. Effects of Nicotinamide and Nicotinic Acid on Activity of 3,3-Dimethyl-1-phenyltriazene in S-180-Bearing Mice. Therapy was given I.P. for 7 days; uniformly, when combined, the vitamin derivatives preceded the triazene.

Agent and dose/kg/day	Avg diam. of tumors, mm	Avg wt change, g	Mortality
Triazene, 100 mm^3	—	—	10/10
" , 50 "	10.1	−4.5	2/10
Nicotinamide, 500 mg	14.8	+ .5	0/10
" " + triazene, 100 mm^3	—	—	10/10
" " + " 50 "	8.3	−2.5	0/10
Nicotinic acid, 500 mg*	15.5	.0	"
" " + triazene, 100 mm^3	7.8	−4.5	3/10
" " + " 50 "	10.1	−2.0	0/10
Control	15.4	.0	"

* Prepared as the sodium salt immediately prior to injection.

tation that the biological activity of the triazene might reflect the alkylating properties of the benzenediazonium ion, experiments were undertaken to learn if marrow depression might characterize triazene intoxication. Groups of non-tumor-bearing mice were treated with intoxicating doses of TEM (1 mg/kg/day) and the triazene (62.5 cm/kg/day) for 5 days. Another group, serving as control mice, received daily injections of peanut oil, the vehicle used for the 2 agents. Although the triazene-treated mice lost more weight (avg –3.1 g) than did those receiving TEM (avg –1.6 g), the total femoral nucleated cell count for the TEM-treated mice was 33% of the control count, whereas the count for triazene-treated mice was 50% of control values.‡ Thus, the triazene did depress bone marrow and this, undoubtedly, contributes to the overall intoxication of the mouse. However, it is distinctly less potent in this respect than is TEM under the conditions of this experiment.

Phillips(10) observed that mice, intoxicated with the triazene, developed a syndrome characterized by unsteady stance and gait, choreaform movements of the head, and retropulsion (the "waltzing" syndrome). The behavior pattern so closely resembled that described by Goldin *et al.*(11) for mice intoxicated with certain alkylating agents that the "swimming test" applied by the latter investigators to detect the presence of this neurological disturbance was applied in this instance. The results were positive. In separate studies, Dagg *et al.*(12) demonstrated a teratogenic response to the triazene in the developing chick embryo which could be prevented by the administration of nicotinamide. The effects of nicotinic acid or its amide were therefore examined in tumor-bearing mice treated with the triazene. The results of a typical experiment are presented in Table V. It is evident tumor-inhibitory activity of the triazene was not altered by prior administration of nicotinic acid or the amide. However, mice treated with nicotinic acid and surviving the 100 cm triazene dose failed to exhibit the "waltzing" syndrome. In other experiments, 2 groups of non-tumor-bearing mice were treated with nicotinic acid (500 mg/kg/day, as the sodium salt) for 3 consecutive days, and a third group received control injections of isotonic NaCl (10 mice/group). One hour after the last intraperitoneal injection, the group injected with NaCl and one of the groups which had received sodium nicotinate received a single injection of the triazene. The dose of the triazene used (180 cm/kg) was one which previously had been shown capable of inducing the swimming deficiency mentioned above within 24 hours in the majority of mice without subsequent lethality. Examined the day following the triazene injection, 70% of the mice which had received isotonic NaCl and the triazene showed the swimming defect (as well as the behavior symptoms of the "waltzing" syndrome) but none of those mice pretreated with nicotinic acid were so affected. There was no change in this situation for the following 3 days of observation. Any relationship between induction of teratogeny and induction of the "waltzing" syndrome by the triazene, other than that both abnormalities may be prevented by nicotinic acid derivatives, is obscure.

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‡ The method of quantitating total nucleated cells of mouse femurs has been perfected at Sloan-Kettering Institute by Dr. Vincenzo Grifoni, a Visiting Fellow from the U. of Milan, Italy. Details of the method and some of its applications will be the subject of a future publication.

Consideration was given the possibility that chemical interaction in the peritoneal cavity between nicotinic acid and the triazene might account for the alteration in biological activity of the triazene. Appearing to negate this possibility were the results of an experiment wherein the nicotinic acid derivatives were administered by oral intubation 45 minutes prior to intraperitoneal injections of the triazene. Doses of agents were similar to those described in Table V and the results were essentially identical. Or, in another experiment with similar end-results, the vitamin derivatives were injected intraperitoneally 60 minutes before doses of 100, 75, and 50 cm/kg/day of the triazene. Finally, *in vitro* experiments were conducted wherein dilute solutions of nicotinic acid and its amide were added to buffered (pH 6.8) aqueous solutions of the triazene. These preparations were examined for possible changes in the ultraviolet absorption patterns. No significant changes were detected, suggesting failure of interaction under these conditions.

It may be adduced from the above that one of the features contributing to intoxication of the Swiss mouse by the triazene is a central nervous system lesion, the overt manifestations of which correspond with those of the genetically recessive characteristics described for Waltzer or Shaker mice(13). These manifestations may be prevented by the prior administration of nicotinic acid. The precise identity of this lesion with that described by Goldin *et al.*(11) remains to be established. Nevertheless, there is similarity between the circumstances leading to development of the lesion under the 2 experimental conditions which tends to support the notion that the triazene, as was found by Golden *et al.* for specific chlorinated tertiary amines, exerts a part of its biological activity by presenting the mouse with 1) an essential structural configuration and 2) an alkylating group. Failure to protect against triazene-induced tumor inhibition with nicotinic acid, however, suggests a bifunctional capacity of the triazene or, possibly a triazene-catabolite, farther removed from the diazonium ion, possessing tumor-inhibitory properties. Indeed, it is clear from the data in Table V that the higher doses of triazene were lethal to some individuals despite the administration of nicotinic acid but none of those animals, though obviously ill, demonstrated overt manifestations of the central lesion prior to terminus; this is further support for perceiving separable activities for the triazene.

Summary. 1. 3,3-Dimethyl-l-phenyltriazene possesses inhibitory activity for growth of the mouse tumor, sarcoma 180. Certain other triazenes have similar activity. 2. Dimethyl-phenyltriazene produces comparable tumor inhibition whether administered orally or intraperitoneally. 3. The triazene induces the "waltzing" syndrome in the Swiss albino mouse used in this study. 4. Nicotinic acid administered prior to the triazene prevents the "waltzing" syndrome but does not modify tumor inhibition. 5. In doses intoxicating to the Swiss mouse, the triazene is a moderate bone marrow depressant.

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Effects of Certain Triazenes on Chick Embryos and on Tumors Explanted to the Chorioallantois.* (22074)

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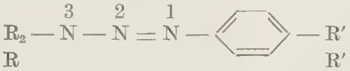
During the screening of various chemical agents for chemotherapeutic activity against the mouse sarcoma 180, it was found that 3, 3-dimethyl-1-phenyltriazene significantly inhibited the growth of this tumor in mice(1). On the basis of the preliminary results against the sarcoma 180, this agent and several related compounds were selected for more extensive study in secondary systems used in the tumor screening program(2-4).

This report is concerned with the effects of certain triazenes on the development of the chick embryo and on tumors growing on the chorioallantois. In the studies reported here it was found that the anomalies induced in the chick embryo by triazenes were similar to those caused by other, unrelated, teratogens whose effects are preventable or reversible by nicotinamide(5-9). Consequently, nicotinamide was tested for its ability to prevent the teratogenic effects of the triazenes.

Materials and methods. Commercially obtained White Leghorn eggs, incubated at 38°, were used for all experiments. The compounds tested for teratogenic activity against the chick embryo have the following structures:



	R	R′
I	CH_3	H
II	CH_3CH_2	"
III	CH_3	$p-C_6H_5-N=N-N(CH_3)_2$
IV	"	C_6H_5
V	"	NO_2

I: 3,3-dimethyl-1-phenyltriazene
II: 3,3-diethyl-1-phenyltriazene
III: 1,1′-(4-4′-biphenylene) bis [3,3-dimethyltriazene]
IV: 1-(4-biphenylyl)-3,3-dimethyltriazene
V: 3,3-dimethyl-1-*p*-nitrophenyltriazene

I is an amber-colored liquid with a specific gravity of 1.02. II is also a liquid, while III, IV, and V are solids. Because of their low solubility, these compounds were prepared as suspensions in isotonic saline or in 0.5% sodium carboxymethylcellulose in isotonic saline. The freshly prepared suspensions were injected into the yolk sac of 4-day-old embryos. The eggs were sacrificed after the 18th day of incubation. The embryos were weighed, examined for gross abnormalities, and selected embryos were cleared and stained with alizarin to show skeletal defects. In the protection experiments, nicotinamide in isotonic saline was injected immediately following the triazene. The tumors used were mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3 (H.Ep.#3)(10,11). Preparatory to chemotherapy trials, triazene-I was injected into the yolk sac of 12-day-old embryos to determine tolerated dose levels

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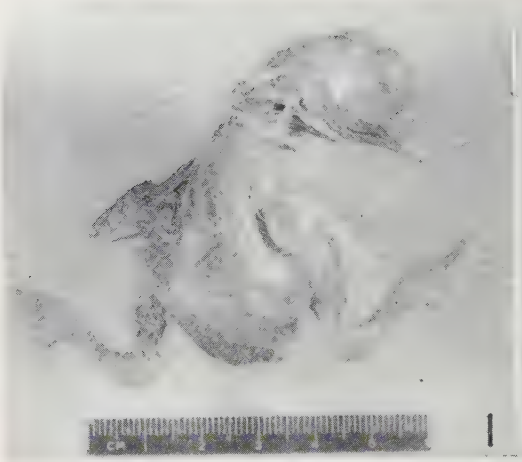
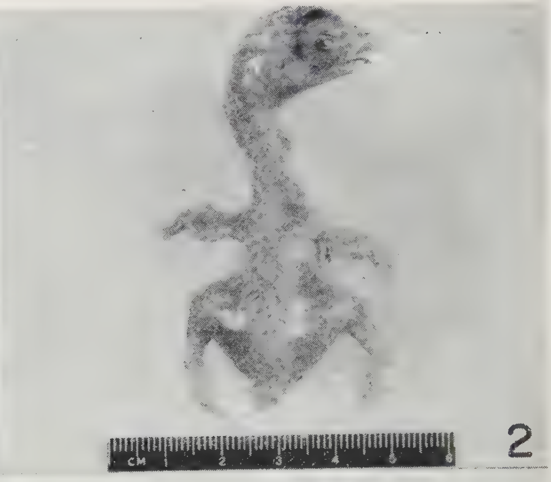
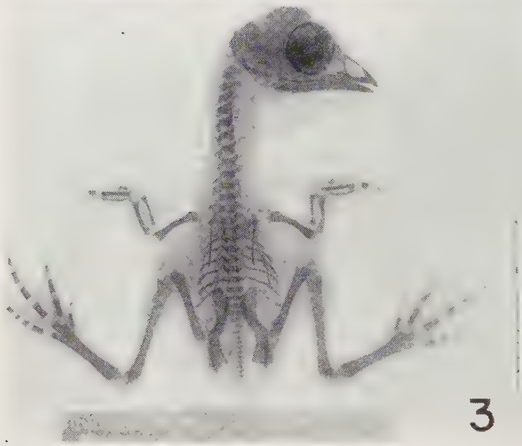
† Public Health Research Fellow of the National Cancer Institute.







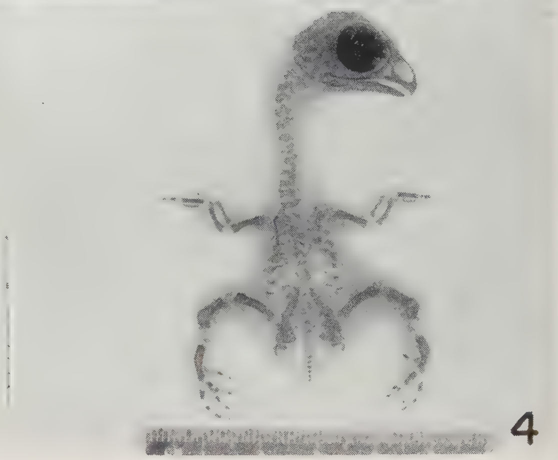


PLATE I

FIG. 1. 18-day control embryo.
FIG. 2. 18-day embryo treated with triazene-I at 4 days.
FIG. 3. Skeleton of 18-day control embryo.
FIG. 4. Skeleton of 18-day embryo treated with triazene-I at 4 days.

and the morphological alterations in the surviving embryos.

Single fragments of tumor were explanted to the chorioallantois on the 8th day of incubation. Four days later, after those eggs with implanted and apparently viable tumors had been selected, triazene-I was injected into the yolk sac. On the 16th day of incubation the eggs were sacrificed; the tumors were weighed and portions saved for microscopic examination. Whenever possible the tumors were bioassayed. Fragments of the H.Ep.#3 were pooled in 100-150 mg lots in each experimental group and inoculated into irradiated-cortisonized rats. Individual tumors of the S180 were assayed by subcutaneous implantation into mice.

Results. Teratogenic activity of 3,3-dimethyl-1-phenyltriazene: Triazene-I, when injected on the 4th day of incubation, produced consistent and characteristic abnormalities in development, clearly seen in embryos surviving to the 18th day of incubation (Fig. 1-4). The LD_{50} was 0.5-1.0 cmm/egg and the teratogenic effects were produced over this range (Table I). The abnormal embryos showed weight inhibition associated with skeletal deformities of the beak and appendages. Edema and feather growth inhibition, with clubbing, occasionally occurred.

TABLE I. Toxicity and Incidence of Abnormal Embryos in Eggs Injected with Triazenes on 4th Day of Incubation and Sacrificed after 18th Day. Protection against teratogenic effects of triazenes with nicotinamide.

	Dose, mm^3/egg	No. inj.	Mortality, %	Abnormal survivors, %
T*-I	.25	20	40	25
	.5	47	30	79
	1.0	25	76	100
	2.0	15	93	100
T-II	.25	20	35	69
	.5	45	49	100
	1.0	20	45	100
	2.0	15	67	100
T-I + N*	.5 5.0	45	62	0
T-II + N	.5 5.0	43	86	0
T-II + N	.5 1.25	49	78	0

* T = Triazene; N = Nicotinamide.

The principal beak defect was parrot-beak (Fig. 2 and 4), although less severe effects such as shortening of the upper and lower beak, or the lower beak alone, were sometimes seen. Abnormalities of the appendicular skeleton included micromelia, bending of the tibiotarsus, and occasionally, bending of the tarsometatarsus. No abnormalities were found in the axial skeleton or in the pelvic and shoulder girdles.

Following injections into the 12-day yolk sac, no gross skeletal abnormalities were produced at tolerated doses. Doses of 5 to 10 cmm/egg led to stunting, inhibition of feather growth with clubbing, and edema in a large percentage of the embryos. Another consistent effect in the 12-day embryo was a reduction in spleen weight. Thus, at 16 days the control spleens weighed 13.6 mg, on the average, as compared to 4.5 mg for the affected embryos.

Teratogenic activity of other triazenes: The tolerated doses and the estimated LD$_{50}$'s of triazenes II, III, IV, and V in the 4-day embryo did not appear to be appreciably different from triazene-I. In this series, only 3,3-dimethyl-1-p-nitrophenyltriazene failed to produce any abnormalities. Triazene-II induced the same pattern of embryonic malformations seen with triazene-I and at approximately the same doses. Triazene-III, however, was effective only at much higher dose levels and affected fewer of the surviving embryos. The overall teratogenic effects of triazene-IV were different from those of the other active compounds in that they occurred less consistently and the only abnormality observed was a bending of the tibiotarsus.

Protection with nicotinamide: Nicotinamide was tested against doses of triazene-I and II which, alone, produced a high incidence of abnormalities in the surviving embryos (Table I). Under these conditions nicotinamide protected against the teratogenic effects but did not restore the weight to normal nor did it protect against the lethality of triazene-I and II. The minimal effective ratio of nicotinamide to triazene has not been determined.

Tumor inhibiting effect of triazene-I: In the chemotherapy tests, triazene-I inhibited the growth of the S180 and H.Ep#3 (Table II). Histological sections of the tumor

TABLE II. Effect of Triazene-I on Mouse Sarcoma 180 and Toolan's H.Ep. #3 Growing on Chorioallantois of the Chick Embryo.

Group	Dose, mm^3/egg	No. inj.	No. survivors	Tumor wt, mg	Embryo wt, g
		Mouse sarcoma 180			
Control	0	8	7	172 ± 72	14.4 ± 1.9
Triazene-I	5.0	10	6	76 ± 44	12.9 ± 1.1
"	7.5	10	6	38 ± 39	10.0 ± 1.9
		Toolan's H.Ep. #3			
Control	0	29	26	229 ± 48	12.7 ± 1.3
Triazene-I	5.0	34	27	42 ± 30	10.0 ± 1.2
"	7.5	24	15	49 ± 37	9.1 ± .7
"	10.0	15	2	19 & 38	8.0 & 8.0

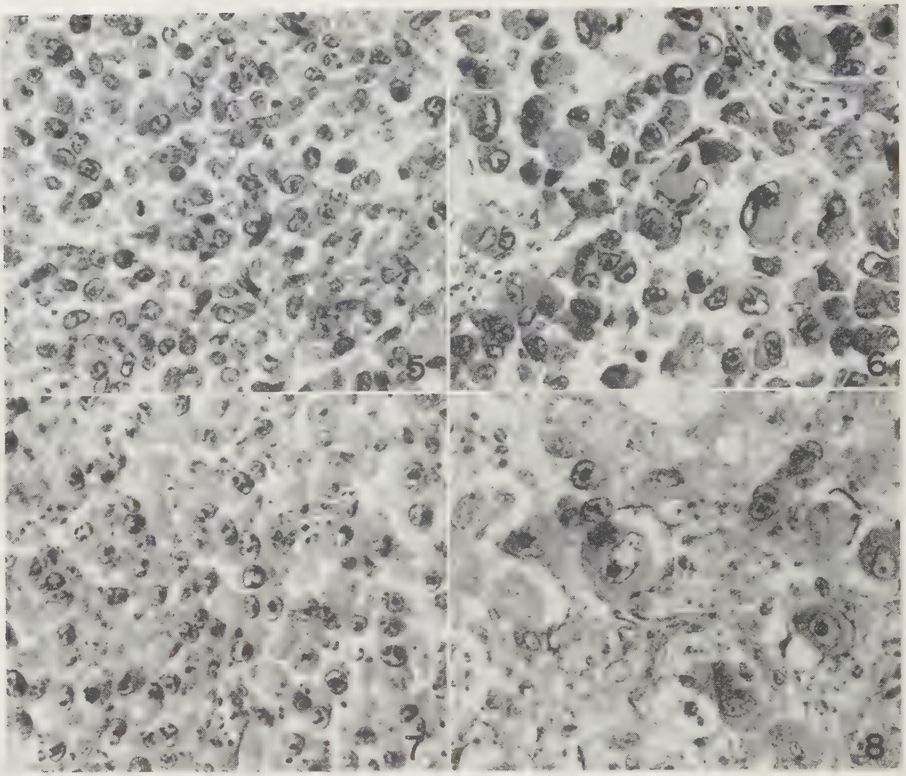


PLATE II

FIG. 5. Untreated mouse sarcoma 180 growing on the chorioallantois. × 200.

FIG. 6. Mouse sarcoma 180 growing on the chorioallantois, treated with triazene-I, 5.0 mm^3 per egg. × 200.

FIG. 7. Untreated Toolan's H.Ep. #3 growing on the chorioallantois. × 200.

FIG. 8. Toolan's H.Ep. #3 growing on the chorioallantois treated with triazene-I, 7.5 mm^3 per egg. × 200.

showed cellular enlargement, nuclear changes indicative of cellular injury, and a decrease in number of cells (Fig. 5-8). Histologically, the sarcoma 180 appeared to be more sensitive than the H.Ep.#3, since greater cellular damage was observed at the lower dose (5 cmm) in the sarcoma 180. When the sarcoma 180's were assayed in mice, the untreated tumors grew rapidly, but, in no case did any of the triazene-treated tumors show any evidence of growth during a 2-week observation period. This indicated that the triazene destroyed the viability of sarcoma 180 as measured by transplantation. In spite of the marked histological effects produced by triazene in the egg-grown H.Ep.#3, the tumors gave positive bioassays in rats. It is noted, however, that in order to prepare a satisfactory inoculum, several human tumors were pooled for bioassay, and therefore, the inclusion of a slightly damaged tumor in the pooled tumor inoculum could produce a positive bioassay. Thus, it is concluded that the viability of every one of the treated H.Ep. #3's was not destroyed by triazene.

Discussion. The effects of triazenes on chick embryos and on explanted tumors have interesting correlations with other agents. Of major significance is the observation that triazene-I causes distinctive and consistent effects on the mouse sarcoma 180 and Toolan's H.Ep.#3 explanted to the chorioallantois. The most important criteria of effect are the histological changes in the tumor and viability on bioassay. The histological changes of cellular enlargement and nuclear distortions with a decrease in cell numbers are similar to those engendered by nitrogen mustard and related polyfunctional alkylating agents and by x-rays(12). The greater sensitivity of the sarcoma 180 as compared to H.Ep.#3 to triazene parallels the results seen with nitrogen mustard(11). A further similarity between the two types of compounds is that both the polyfunctional alkylating agents and triazene-I retard the growth of the embryonic spleen.

Quantitative dissimilarities between triazenes and polyfunctional alkylating agents are apparent in their comparative effects on 12-day-old chick embryos and egg-grown tumors. Doses of nitrogen mustard causing relatively slight disturbances in the embryo are markedly injurious to the tumors and affect their viability as indicated by bioassay (12,13). In contrast, the dose of triazene-I required to obtain an equivalent degree of tumor inhibition and cellular damage will induce definite injury to the embryo.

Despite certain similarities in the biological actions of nitrogen mustards and the triazenes, parallel information on the chemical reactivity of triazenes in biological systems is not available. It has been shown that among biologically active alkylating agents, compounds possessing 2 functional groups are far more active than the monofunctional agents (one-armed mustards)(14,15). While the amount of triazene necessary to produce effects in the explanted tumors in the egg are much greater than that required for most polyfunctional alkylating agents, 0.1 mg for nitrogen mustard as compared to 5 mg (5 cmm) for triazene-I, neither altering the structure of the triazenes (cf. triazene-I, II, IV, V) nor producing a two-armed compound (triazene-III) has enhanced the biological activity.

The triazenes are of further interest since certain members of this series produce a definite syndrome of malformations when injected into 4-day-old embryos. The effects on the facial and appendicular skeleton are similar, if not identical, to those following treatment by insulin(6,7), sulfanilamide (5,9), and eserine(5,8). Furthermore, as in the case of the latter compounds, nicotinamide will prevent the teratogenic action of triazenes in the 4-day embryo. This teratogenic property distinguishes triazenes from the polyfunctional alkylating agents which do not produce specific or consistent teratogenic effects. The biochemical mechanism for the production of the parrot beak-micromelia-bent tibiotarsus syndrome has not been definitely established, although Landauer(7) and Zwilling(9) have related it to interference with carbohydrate metabolism.

Since the effects of triazene-I on tumors and 12-day embryos are similar to those produced by certain polyfunctional alkylating agents while the effects on younger embryos resemble those following treatment with insulin, sulfanilamide and eserine, it is possible that the molecule is pluripotent or that it is converted in the egg into 2 or more metabolites with dissociated activity. In acid solutions triazene-I is broken to the diazonium salt and dimethylamine. Diazonium salts have been shown to polymerize *in vitro* to form diazoaminobenzene, a compound that is toxic to the liver, spleen, and kidneys in mice, and is carcinogenic in mice on local application to the skin(16). When injected into the 4-day-old embryo, *p*-diphenylamine diazonium fluoroborate produces a syndrome of abnormalities which is similar to that caused by triazenes-I and II(3).

Because of the wide range of biological effects of the polyfunctional alkylating agents, the discovery of a tumor-inhibiting drug with somewhat similar but also distinct effects, and acting by means of a considerably different chemical structure, becomes of interest.

Summary. 1. A new compound that inhibits the growth of the sarcoma 180 in mice, 3,3 - dimethyl - 1 - phenyltriazene, "triazene," also exhibits teratogenic activity in the chick embryo and damages mouse and human tumors explanted to the chorioallantois of the chick embryo. 2. The teratogenic syndrome produced by the injection of "triazene" into the yolk sac of the 4-day embryo consists of parrot-beak, micromelia, bent tibiotarsus, edema, and feather growth inhibition. This effect can be prevented by the injection of nicotinamide. Several analogs of "triazene"

have been tested for teratogenic properties. 3. When injected into the yolk sac of embryos carrying chorioallantoic grafts of the mouse sarcoma 180 and Toolan's human epidermoid carcinoma #3, "triazene" causes inhibition of tumor growth and cellular injury. This effect is similar to that produced by nitrogen mustard and other polyfunctional alkylating agents. Because "triazene" shows certain similarities in tumor-inhibiting activity when compared to nitrogen mustard and because of its distinctly different chemical structure, it warrants consideration as a candidate agent for clinical cancer chemotherapy.

We wish to express our appreciation to Dr. Christian S. Rondestvedt, Jr., of the University of Michigan for supplying several of the triazenes, and to Dr. Helene W. Toolan, of the Sloan-Kettering Institute for conducting the bioassays of the H.Ep. #3.

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Serum Glycoprotein Concentrations in Rats Infected with the Cestode, *Hymenolepis diminuta.* (22075)

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(Introduced by Charles M. Carpenter.)

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The serum protein response in a large number of infectious diseases has been studied by many investigators employing electrophoretic or salting-out methods. Gutman(1), Fisher(2) and Stauber(3) have summarized the significant data in their reviews. In contrast, few reports are available on effects of infectious diseases on carbohydrate-containing proteins of serum. Studies concerned with the influence of infection on protein-bound carbohydrates of serum have been briefly reviewed by Weimer *et al.*(4). Diseases in which serum glycoproteins have been determined are characterized by invasion of tissues by the etiologic agent and marked, systemic host reactions. It was of interest to investigate the serum glycoproteins in an experimental infection in which the parasite is localized and invasion of the tissues does not occur.

The present investigation was undertaken to study the effects of experimental infection with *Hymenolepis diminuta* on serum concentrations of total glycoprotein, seromucoid (mucoprotein), γ-globulin polysaccharide, total protein, and γ-globulin protein in rats.

Hematocrit values and concentrations of hemoglobin were also determined in oxalated blood specimens.

Materials and methods. Twenty male and 7 female adult rats of the Long-Evans strain with an average weight of 305 g were infected. Thirteen male and 7 female rats served as controls. Tapeworm eggs were recovered from the feces of rats infected with *H. diminuta,* Rice Institute strain. Cysticercoids were obtained by feeding the eggs to laboratory-reared beetles, *Tenebrio molitor.* The rats were lightly anesthetized with ether and 5 cysticercoids were administered *per os* with a pipette. The animals were housed 2 per cage and were maintained on a diet of Purina Laboratory Chow and tap water, *ad libitum.* Thirty-two days after inoculation the infected rats were sacrificed by exsanguination from the heart and air embolism. The animals were autopsied and the tapeworms counted. Blood samples were obtained from the normal animals by cardiac puncture. Two ml of each of the blood specimens were added to tubes containing a dried mixture of potassium and ammonium oxalates. The remainder of the blood sample was allowed to clot and the serum separated. Total serum glycoprotein, seromucoid, γ-globulin polysaccharide, total serum protein, and γ-globulin protein were determined by methods previously reported(4,5). Hematocrit values were determined in Wintrobe tubes by the procedure outlined by Kolmer(6). Hemoglobin was determined by an acid-hematin method in a Klett-Summerson photoelectric colorimeter (6). The mean, standard error of the mean, t, and probability values were determined by standard statistical methods(7).

Results. A summary of the data is presented in Table I. No significant differences occurred between the normal and the infected group for the serum determinations. The increases in the hemoglobin and hematocrit values were significant at the 5% level.

TABLE I. Summary of Results.

Determination	Normal (20)	Infected (27)
Wt at bleeding (g)	343 ± 14.0*	345 ± 13.8*
No. of worms		3.9 ± .2
Total serum glycoprotein (mg %)	138 ± 2.0	143 ± 2.0
Seromucoid (mg %)	18 ± 1.2	18 ± .9
γ-globulin polysaccharide (mg %)	31 ± 1.7	30 ± .6
Total serum protein (g %)	6.6 ± .12	6.6 ± .9
γ-globulin protein (g %)	1.9 ± .06	1.9 ± .06
Hemoglobin (g %)	14.6 ± .4	16.1 ± .4
Hematocrit (%)	44.5 ± 1.2	47.7 ± .8

* Stand. error of the mean.

The lack of effect of a well established infection with *H. diminuta* on the serum glycoproteins and proteins of the rat is in marked contrast to changes observed in other pathologic states of infectious etiology. The increase in hematocrit and in hemoglobin values might suggest a stimulation of the hemapoietic system, or reflect loss of water and plasma protein. Further studies are indicated.

Several factors have been suggested as possible causes for the increased concentrations of serum glycoproteins in a wide variety of pathologic and physiologic states: tissue degradation(8), tissue proliferation and repair(9), protein synthesis(10), and nonspecific stress(11). Regardless of the cause of elevated serum glycoprotein levels, it is apparent that none of the responsible factors was operative in the present investigation.

It may be inferred from the results reported above, that a localized infection without tissue invasion has no significant effect on the level of the serum glycoproteins and proteins of the rat.

Summary. The effects of experimental infection with *Hymenolepis diminuta* on the serum concentrations of total glycoprotein, seromucoid, γ-globulin polysaccharide, total protein, γ-globulin protein and on hemoglobin and hematocrit values have been determined in Long-Evans rats. No statistically significant differences occurred in serum values between the normal and the infected groups. An increase in hemoglobin and hematocrit values, significant at the 5% level, was observed in the infected group.

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Role of Hyperlipemia in the Genesis of Hypercholesteremia.* (22076)

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It has long been known that an hypercholesteremia, either occurring clinically or induced experimentally, is usually accompanied by a rise in the concentration of plasma phospholipid and sometimes also of neutral fat. This frequently observed association of hypercholesteremia with hyperlipemia has not been investigated in respect to the possible *sequential* relationship of these multiple lipid rises. Instead it has been more or less assumed, in lieu of direct evidence, that the lipid rises observed in hypercholesteremia are merely associative phenomena. Recently, however, Allbrink, Man and Peters(1), noting the physical association of plasma cholesterol with neutral fat in chylomicra, have posed the question of whether an excess of plasma neutral fat might not dissolve additional cholesterol thus preventing its inclusion in soluble lipoprotein, leading thereby in some manner to a state of hypercholesteremia.

In view of this last possibility, it was decided to induce a sustained state of hyperlipemia in the rat by continuous parenteral injection of 2 types of lipid mixture (containing only neutral fat and lecithin) and then observe the possible changes in plasma cholesterol resulting therefrom. The results obtained in this study, as herein reported, indicate that a state of hypercholesteremia quickly follows the induction of a prolonged hyperlipemia.

Materials and methods. Male rats (Long Evans strain) 12-16 weeks of age were used in all of the experiments. (a) Production of sustained hyperlipemia was accomplished in rats by rapid intravenous administration of 3 ml of either of 2 different lipid emulsions (B† and Z‡) followed by *continuous* intravenous administration of the same emulsion at 0.9 to 1.2 ml/hour for 12 hours. This continuous intravenous administration to an unanesthetized rat was made possible by its enclosure in the Bollman type of cage(2). Prior to the experiment these rats had been placed for 7-15 days on a diet comprised of Purina Chow enriched with cholic acid (2%) and olive oil (2%). Each rat also received an additional 3 ml of olive oil by stomach tube at 72, 48 and 24 hours prior to the start of experiment. All food was withheld from rats for 24 hours immediately prior to and during the experi-

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† B lipid emulsion (kindly furnished by Don Baxter) is composed as follows: Sesame Oil, U.S.P. 10; Dextrose, U.S.P. 4.5; Purified Soya Bean Lecithin 0.65; Water, q.s. 100 ml. Lipid is present as finely divided particles of 1 μ or less in diameter.

‡ Z lipid emulsion (kindly furnished by D. B. Zilversmit of University of Tenn.) is composed as follows: Coconut Oil 10; Purified Soya Bean Lecithin 1.0; Dextrose 5.0; Water, q.s. 100 ml. Lipid particles are approximately the same diameter as in B emulsion.

ment. This pre-feeding regimen was adopted after preliminary studies had demonstrated that the usual rapid decline in hyperlipemia after a single injection of a fat emulsion could be slowed if the animal's fat absorption previously had been enhanced. Plasma samples obtained before, immediately after the initial injection of 3 ml of fat emulsion, and 12 hours after beginning of continuous intravenous injection, were analyzed for cholesterol(3) and phospholipid(4). The total lipid of the samples was analyzed by the method of Bragdon(5). Control animals were fed same regimen but received glucose solution (5%) intravenously in lieu of fat emulsion. (b) *Partial hepatectomy.* Three animals were prefed and later administered lipid emulsion as described above except that just prior to lipid administration, the rats were anesthetized with ether and a portion (approximately 2.7 g) of liver removed. (c) *Production of hyperlipemia by injection of protamine.* Freshly prepared protamine sulfate solution (salmine)§ solution (2 mg in 3 ml normal saline solution) was injected into a series of rats given 3 ml of olive oil just prior to injection and into a control series fasted for 24 hours. Both series were given additional intravenous injections of 2 mg of protamine at 3, 6 and 9 hours following initial injection. For additional control purposes, a third series of rats were given 3 ml of olive oil and injections of dextrose solution (5%) comparable in volume to the volumes injected in the first 2 series. Plasma samples obtained before and 12 hours after the initial injection were analyzed for cholesterol and total lipid. (d) *In vitro extraction of cholesterol from hypercholesteremic serum by lipid suspension.* Pooled hypercholesteremic but non-turbid serum (cholesterol concentration: 128 mg/100 ml) was obtained from bile ligated, cholate fed rats(6). A lipid suspension was obtained as follows: The 24-hour intestinal lymph of 5 rats fed 3 ml of olive oil was collected and submitted to ultracentrifugation (r.c.f. = 13,000-27,000) for 30 minutes. The resulting top creamy layer was removed and suspended in 5 ml of H_2O. This

TABLE I. Total Lipid and Cholesterol Response of Intact and Partially Hepatectomized Rat to Continuous Injection of Lipid Emulsion.

Rats			Blood plasma								
			Total amt	Total lipid (mg/100 ml)			Phospholipid (mg/100 ml)			Plasma cholesterol (mg/100 ml)		
No.	Given	Avg wt (g)	inj. (ml)	A	B	C	A	B	C	A	B	C
10	Control rats, dextrose sol.	301 (282–314)	12.5 (11.8–13.3)	286 (147–286)	—	219 (185–263)	101 (90–124) ± 2.3	—	125 (107–142) ± 3.5	62 (52–76) ± 2.24	—	80 (69–96) ± 2.44
13	B fat	299 (235–368)	15.2 (13.8–16.4)	189 (131–292) ± 13.2	—	681 (500–958) ± 53.0	97 (81–114) ± 4.8	185 (93–310) ± 38	196 (120–271) ± 15.4	53 (35–78) ± 3.4	60 (48–80) ± 2.4	124 (80–195) ± 11.2
5	Z fat	318 (304–328)	15.2 (14.8–15.4)	177 (138–296) —	1888	864 (604–1140) —	121 (100–176) —	198 (184–238) —	251 (181–327) —	58 (48–70) —	62 (51–67) —	128 (88–180) —
3	Partial hepatectomized rats, B fat	310 (302–318)	14.6 (14.0–15.2)	190 (181–200) —	—	562 (523–597) —	—	—	—	50 (47–51) —	54 (45–64) —	69 (67–73) —

A = Before inj. B = Immediately after initial inj. C = 12 hr after inj.
Values in parentheses = Range; ± values = Stand. error of the mean.

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§ Eli Lilly and Co. control No. 8367-571094 DH.

TABLE II. Total Lipid and Cholesterol Response of Intact Rat to Intermittent Injections of Protamine.

Rats			Blood plasma			
			Total lipid (mg/100 ml)		Plasma cholesterol (mg/100 ml)	
No.	Given	Avg wt (g)	A	B	A	B
11	Protamine	311 (292–340)	138 (121–152) ± 5.9	238 (175–324) ± 21.4	48 (33–65) ± 3.6	60 (38–77) ± 3.4
9	Protamine & olive oil	325 (300–344)	204 (151–273) ± 20.3	563 (263–1390) ± 66.0	56 (39–78) ± 4.0	119 (96–174) ± 7.9
9	Olive oil	310 (302–318)	218 (148–356) ± 22.4	287 (210–394) ± 54	50 (47–51) ± 3.8	69 (67–73) ± 7.4

A = Before inj. B = 12 hr after inj.
Values in parentheses = Range; ± values = Stand. error of mean.

latter suspension was considered to consist almost entirely of a mixture of chylomicronous lipids, relatively free of protein(7) and had a cholesterol concentration of 148 mg and a total lipid concentration of 4440 mg/100 ml. Two ml of the lipid suspension were mixed with 2 ml of hypercholesteremic serum, shaken at room temperature for 6 hours and then the mixture ultracentrifuged. The resulting top creamy layer (approximately 0.9 ml) was removed, suspended in 5 ml of water and analyzed for cholesterol. The lower layer also was similarly analyzed. For control purposes, the identical procedure was repeated substituting a solution of H_2O for the hypercholesteremic serum.

Results. As Table I indicates, a continuous state of hyperlipemia characterized by a rise in both total lipid and phospholipid content of rat's plasma, was obtained by continuous injection of either B or Z lipid emulsion. Induction of this hyperlipemia resulted in a marked rise in cholesterol content of plasma. Thus when hyperlipemia was induced by injection of B emulsion, the average plasma cholesterol content increased from 53 mg before to 124 mg/100 ml in 12 hours, a rise of 134%. Approximately similar results in respect to cholesterol were obtained following parenteral administration of Z emulsion (See Table I), although its injection led to a greater elevation of plasma lipid, possibly due to its greater phospholipid content. Continuous injection of dextrose solution into control animals did not elevate their total plasma lipid but a moderate rise in their plasma phospholipid (24%) and cholesterol (29%) occurred. These latter rises however cannot be ascribed to administration of dextrose solution because they invariably occur in fat fed rats following any type of experimental procedure (*e.g.,* routine bleeding for samples).

Induction of lipemia in partially hepatectomized rats did not lead to a significant rise in plasma cholesterol. This failure of cholesterol to rise in partial absence of the liver suggests that the excess cholesterol observed in plasma of above intact rats after induced lipemia came from a hepatic and not an intestinal source. Starvation of all intact animals 24 hours prior to experimental procedure also suggests a source other than the intestine of the excess cholesterol.

The results following injection of protamine also suggested that experimental induction of lipemia alone induces a cholesteremia. Thus (Table II) although *intermittent* injection of protamine into the fasted rat produced a possible moderate increase in total lipid, it did not induce marked hypercholesteremia. However when the same dosage of protamine was given to rats fed olive oil, a significant hyperlipemia occurred and a hypercholesteremia (Table II). These results are in agreement with those found by Szasz and Constantinides(8).

In vitro exposure of pooled hypercholesteremic serum to the suspension of intestinal lymph lipids resulted in a fall of cholesterol content of serum from 2.56 to 1.0 mg and a corresponding rise in cholesterol content of chylomicron lipids from 2.4 to 3.4 mg. The cholesterol content of control chylomicron lipid mixture remained unchanged. In other words, the lipid mixture was capable of extracting over half of the cholesterol originally present in clear hypercholesteremic serum.

Discussion. Our findings indicate that hypercholesteremia resulted whenever a sufficient degree of sustained hyperlipemia was induced by the methods employed. Furthermore, it appears that this cholesterol rise will occur regardless of whether the hyperlipemia ensues, because too much lipid is presented by parenteral administration to the organism for its complete removal from blood, or because the organism is prevented by protamine injection from removing its usual amount of lipid from blood. Cholesterol thus retained also originates primarily in the liver. Apparently a portion of the cholesterol normally discharged into plasma and then later taken up again by the liver is "trapped" in the excess lipid circulating in plasma. Ability of a lipid suspension derived from intestinal lymph to extract cholesterol existing in soluble, ostensibly lipoprotein form(9) in hypercholesteremic serum further suggests the possibility of such "trapping." Apparently then lipid can dissolve cholesterol existing in plasma, as Allbrink, Man and Peters(1) have suggested, and this occurs even when cholesterol is present in soluble lipoprotein bondage.

While previously hypercholesteremia and hyperlipemia have been considered independent processes, the demonstration that a rise in plasma lipid quickly leads to hypercholesteremia suggests that it is necessary to consider the possibility that an excess rise of cholesterol in plasma (when attended by hyperlipemia) may be passively induced resultant of *primary* derangement in plasma lipids other than cholesterol. This induced hypercholesteremia could have been effected by accumulation in plasma of either neutral fat, phospholipid or both lipids present in the emulsions. Present studies are in progress to determine the magnitude of particular responsibility of each of these other lipid species.

Summary. Induction of hyperlipemia in rats by continuous intravenous injection of lipid mixtures containing only neutral fat and soya lecithin quickly led to hypercholesteremia. These results suggest that in hypercholesteremia associated with hyperlipemia, the primary derangement may lie not in the cholesterol but in some phase of the fat metabolism.

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Chlorpromazine and Anesthetic Agents on Tissue Non-Protein Sulfhydryl Compounds in Normothermic and Hypothermic Rats.* (22077)

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Previous work(1,2) has shown that cold and restraint lower the concentration of non-protein sulfhydryl compounds (NPSH). Adrenalin also decreases liver and kidney NPSH (3) and it was later observed that some anesthetics alter liver NPSH. To determine the effect of anesthetics and chlorpromazine on tissue NPSH and the effect of cold on tissue NPSH of anesthetized animals, the NPSH in liver and kidney was measured on normothermic and hypothermic rats.

Methods and materials. The 210 adult Sprague-Dawley female rats were divided into the following groups: 52 control animals; 18 animals were given chlorpromazine hydrochloride; 17 were anesthetized with ether; 14 with nitrous oxide, 16 with phenobarbital sodium (phenobarbital), 39 with pentobarbital sodium (nembutal), 38 with thiopental sodium (pentothal), and 16 with thiamylal sodium (surital). Each group of treated animals was divided into 2 categories. Approximately one-half of the animals in each group was maintained at normal body temperature while the remaining animals were exposed in a cold room (0°C ± 1°C) and body temperature fall was regulated at 5°C per hour. All exposures were 4 hours in duration so that the body temperature of the hypothermic animals was approximately 18°C at the time of sacrifice. Chlorpromazine was injected intraperitoneally. These animals were in very light anesthesia exhibiting some reflex response to deep pain. Animals exposed to gaseous anesthetics (ether and nitrous oxide) were placed in a large glass chamber and the anesthetics and oxygen admitted at a rate to keep animals in very light surgical anesthesia. If animals were more deeply anesthetized, they would not survive the 4-hour exposure. Animals anesthetized with the barbiturates were injected intraperitoneally with phenobarbital, 20 mg/kg; thiamylal, 25 mg/kg; pentobarbital, 70 mg/kg; thiopental, 75 mg/kg initially, and thereafter sufficient to keep the animal in a relaxed state. In the barbiturate anesthesia experiments, the animals were maintained in surgical plane of anesthesia. The animals were then sacrificed and the organs removed immediately and frozen in dry ice. The NPSH was measured by a modification of the method of Benesch and Benesch(4).

Results. As may be seen from Table I, all anesthetized animals experienced a drop in liver NPSH. In the groups anesthetized with phenobarbital, thiamylal and thiopental, there was a significantly greater drop in the hypothermic categories than in the normothermic categories. However, in the groups given chlorpromazine, ether, nitrous oxide, and pentobarbital, exposure to cold did not have the usual effect of further decreasing the liver NPSH.

Ether, nitrous oxide, phenobarbital, and pentobarbital produced a significant decrease in kidney NPSH. Thiamylal and chlorpromazine had no effiect; whereas thiopental produced a slight increase. Cold had no effect on the kidney NPSH of animals given ether, nitrous oxide, phenobarbital and thiamylal. The thiopental and chlorpromazine groups exposed to cold showed a decrease in kidney NPSH.

Thiopental appeared to increase the blood NPSH (105 ± 7.1 μM% to 133 ± 4.8 μM%, $P = < .05$). No significant change in blood NPSH was observed when chlorpromazine hydrochloride was administered.

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TABLE I. Effect of Chlorpromazine and Anesthetics on Liver and Kidney Non-Protein Sulfhydryl Compounds in Normothermic and Hypothermic Rats.

Treatment	Body temp., °C	No. of animals	μM % NPSH in liver	Probability† With control	Probability† Hypothermic with normothermic	μM % NPSH in kidney	Probability With control	Probability Hypothermic with normothermic
Control	39	52	852 ± 7*			508 ± 6		
Chlorpromazine hydrochloride	20	9	567 ± 15		<.20	396 ± 17		<.01
	39	9	604 ± 21	<.01		499 ± 18	<.50	
Ether	18	10	693 ± 17		<.10	421 ± 17		>.50
	39	7	619 ± 28	"		432 ± 28	<.05	
Nitrous oxide	18	8	579 ± 21		"	406 ± 21		>.40
	39	6	488 ± 24	"		404 ± 11	<.01	
Phenobarbital sodium	18	8	597 ± 24		<.01	500 ± 24		>.05
	39	8	705 ± 13	"		450 ± 17	<.02	
Pentobarbital sodium	18	19	636 ± 12		>.10	401 ± 35		>.10
	39	20	655 ± 20	"		341 ± 23	<.01	
Thiopental sodium	18	20	540 ± 15		<.01	415 ± 10		<.01
	39	18	658 ± 18	"		551 ± 17	<.05	
Thiamylal sodium	18	8	582 ± 19		"	512 ± 16		>.50
	39	8	679 ± 16	"		516 ± 17	>.50	

* Stand. error of the mean.

† P is probability derived from t values where $t = \frac{\text{Difference between means}}{\text{Stand. error of difference}}$.

Discussion. Hypothermia and stress depress metabolism in rats(5) and also decrease NPSH in actively metabolizing tissues(1,2). Since many anesthetic agents depress metabolism and produce a tendency toward hypothermia, it is not surprising that anesthetics decrease tissue NPSH. Adrenalin has been shown to lower the tissue NPSH(3). The sympatho-adrenal system is activated by most anesthetics(6), thus the lowered levels of the liver NPSH noted in these experiments may have been due to the activation of this system.

The usual effect of cold of lowering the liver NPSH was observed in the animals anesthetized with phenobarbital, thiopental, and thiamylal. However, it appears that chlorpromazine and pentobarbital may have paralyzed the mechanism activated by cold which decreases liver NPSH. Most anesthetics which caused a lowering of kidney NPSH also prevented any further diminution by exposure to cold. The only exceptions were chlorpromazine and thiopental. Since thiopental contains a sulfur atom, it is possible that the higher kidney levels in the normothermic category may represent concentrations of sulfhydryl groups from the anesthetic in the excretory process. Increased blood levels of NPSH were also observed when this drug was given. Since thiamylal was given at only one-third the level of thiopental, this slight effect would not be seen. The decrease in kidney NPSH in the hypothermic thiopental and chlorpromazine groups is essentially the same effect as that observed when control animals were exposed to cold and restraint(7).

Summary. 1. Female rats were divided into groups and given chlorpromazine or anesthetic agents. One-half of each group was exposed to cold. The total non-protein sulfhydryl concentration (NPSH) of liver and kidney was determined and compared with similar values for control animals. Chlorpromazine and all anesthetics produced a decrease in liver NPSH. Cold further decreased the NPSH of animals anesthetized with phenobarbital, thiamylal, and thiopental; however, cold had no effect on liver NPSH of the chlorpromazine and pentobarbital groups. 2. Ether, nitrous oxide, and phenobarbital de-

creased kidney NPSH. No change was observed in the chlorpromazine and thiamylal groups, but a slight increase in kidney NPSH occurred in the thiopental group. The possible relationship of anesthetics on tissue NPSH and metabolism is discussed.

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Effect of Administered Ascorbic Acid on Nitrogen Metabolic Response to Thermal Trauma. (22078)

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Numerous investigators have confirmed Cuthbertson's(1) observations of an increase in urinary nitrogen excretion by injured patients and animals. Lowered excretion of ascorbic acid, decreased adrenal and plasma ascorbic acid content, and decreased tissue ascorbic acid "saturation" as judged by load tests have also been observed during the period of increased urinary nitrogen excretion (2-5). These changes in ascorbic acid and nitrogen metabolism may be interrelated; ascorbic acid may be important in adrenal cortical function, and there is evidence to indicate that the nitrogen metabolic changes after injury are dependent, in part, on the level of adrenal activity. Ascorbic acid administered subcutaneously to normal rats was shown by Noble and Toby(6) to reduce urinary nitrogen excretion for at least 34 hours, the period of their observations. Further, rats traumatized by leg clamping and given ascorbic acid, either subcutaneously or orally, excreted less nitrogen in the first 34 hours after injury, the period of their observations, than rats similarly injured but not given ascorbic acid.

Accordingly, we have investigated the effects on nitrogen excretion of supplemental ascorbic acid given to rats with severe thermal injury throughout the period of negative nitrogen balance (2½ weeks following injury).

Methods. Thirteen male young adult albino rats of the Wistar strain were adapted over a period of 6 days to the force-feeding of a liquid diet(7,8). From the end of the adaptation period to the end of the experiment, each of the animals was given 10 cc of the diet 2 times daily. According to chemical analysis, each animal received 640 mg of nitrogen every 2 days. By calculation, this diet yielded about 0.2 calorie per gram of body weight per day and was made up, on a caloric basis, of 18% protein, 63% carbohydrate, and 19% fat. Six days after rats reached full feeding, they were placed in metabolism cages from which acidified urine and feces were collected every 2 days. Total nitrogen in urine, feces, and diet was determined by micro-Kjeldahl analysis(9). Urinary amino nitrogen was estimated by the colorimetric ninhydrin technic(10); no correction was made for urea which reacts with ninhydrin to the extent of 3%. Rats were weighed every other day. After a 12-day control period, all rats were lightly etherized and deep thermal injuries produced over 30-35% of their body surfaces by dipping their clipped backs into hot water (73°C) for 30 seconds

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TABLE I. Nitrogen Metabolism in Thermally Injured Rats: Effect of supplemental ascorbic acid.

Day of study	Body wt (g)		Urinary amino N (mg/48°)†		Urinary total N (mg/48°)†	
	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented
1- 2	295 ± 6*	278 ± 9	29.9 ± 2.1	30.7 ± 3.9	534 ± 48	504 ± 34
3- 4	286 ± 8	271 ± 8	32.5 ± 2.0	31.1 ± 4.1	515 ± 46	499 ± 43
5- 6	287 ± 8	274 ± 8	31.8 ± 1.7	29.3 ± 1.3	502 ± 24	491 ± 32
7- 8	293 ± 7	278 ± 7	29.7 ± 4.1	31.5 ± 4.1	500 ± 23	482 ± 48
9-10	293 ± 6	279 ± 8	(lost)	(lost)	513 ± 60	475 ± 28
11-12	296 ± 5	282 ± 7	31.6 ± 3.7	34.9 ± 2.6	506 ± 56	519 ± 52
13-14‡	296 ± 5	283 ± 7	37.5 ± 2.6	41.3 ±10.8	700 ± 98	702 ± 102
15-16	307 ± 9	297 ± 13	34.0 ± 4.7	37.2 ± 6.2	704 ± 88	718 ± 106
17-18	298 ± 11	279 ± 10	40.8 ± 2.8	40.8 ± 2.1	741 ± 43	746 ± 48
19-20	287 ± 10	267 ± 7	39.3 ± 3.8	43.5 ± 5.0	698 ± 77	725 ± 74
21-22	282 ± 9	261 ± 9	37.1 ± 3.1	41.3 ± 4.1	678 ± 35	733 ± 81
23-24	276 ± 11	260 ± 9	39.3 ± 6.3	42.7 ± 6.9	633 ± 75	624 ± 91
25-26	272 ± 12	254 ± 11	44.3 ± 6.9	38.4 ± 7.2	716 ± 102	609 ± 101
27-28	270 ± 19	245 ± 22	41.0 ± 4.9	47.1 ± 6.5	697 ± 107	695 ± 58
29-30	260 ± 11	250 ± 14	35.4 ± 8.9	37.1 ± 5.9	603 ± 117	581 ± 32

* Stand. dev. † 48° intake, 640 mg N (constant). ‡ All rats thermally injured on day 13.

according to the method of McCarthy(11). All rats were maintained on the constant tube-fed diet. Seven of the 13 animals received supplemental ascorbic acid; the first dose (25 mg) was given intramuscularly 1½ hours after injury. Thereafter, 25 mg of ascorbic acid were given twice a day, orally, to each of these 7 rats. Ascorbic acid was added to their food just prior to each tube feeding. The study was conducted for 18 days after injury, by which time the nitrogen excretion in both groups had returned close to the pre-injury levels. At this time the injured skin of the back was in the form of a dried eschar; epithelization was slowly taking place at the periphery of the injured area.

Results. During the control period, all rats were in slight positive nitrogen balance. Their body weights were stable. The thermal injury was followed by a loss in body weight (after a transient gain due to edema in the injured area) and prompt and prolonged increase in urinary nitrogen excretion, total and amino. These changes were uninfluenced by the administration of ascorbic acid (Tables I and II; Fig. 1). (The data were analyzed

TABLE II. Nitrogen Metabolism in Thermally Injured Rats: Effect of supplemental ascorbic acid.

Day of study	Urinary + fecal total N (mg/48°)†		N balance (mg/100 g body wt/48°)†	
	Controls	Ascorbic acid supplemented	Controls	Ascorbic acid supplemented
1- 2	594 ± 45*	557 ± 40	+16 ± 15	+30 ± 14
3- 4	576 ± 35	551 ± 43	+23 ± 12	+33 ± 15
5- 6	552 ± 29	541 ± 38	+30 ± 10	+36 ± 13
7- 8	550 ± 16	547 ± 42	+31 ± 6	+33 ± 14
9-10	560 ± 26	524 ± 32	+27 ± 8	+42 ± 12
11-12	561 ± 50	568 ± 50	+27 ± 16	+26 ± 16
13-14‡	746 ± 108	749 ± 101	−36 ± 36	−39 ± 33
15-16	750 ± 93	766 ± 106	−36 ± 31	−42 ± 35
17-18	797 ± 47	811 ± 52	−53 ± 15	−61 ± 16
19-20	754 ± 105	789 ± 76	−40 ± 35	−56 ± 25
21-22	750 ± 25	799 ± 85	−39 ± 9	−61 ± 28
23-24	672 ± 67	690 ± 90	−12 ± 22	−19 ± 30
25-26	751 ± 98	669 ± 110	−41 ± 33	−11 ± 36
27-28	757 ± 103	761 ± 56	−43 ± 34	−49 ± 18
29-30	623 ± 95	636 ± 32	+ 7 ± 32	+ 2 ± 11

* Stand. dev. † 48° intake, 640 mg N (constant). ‡ All rats thermally injured on day 13.

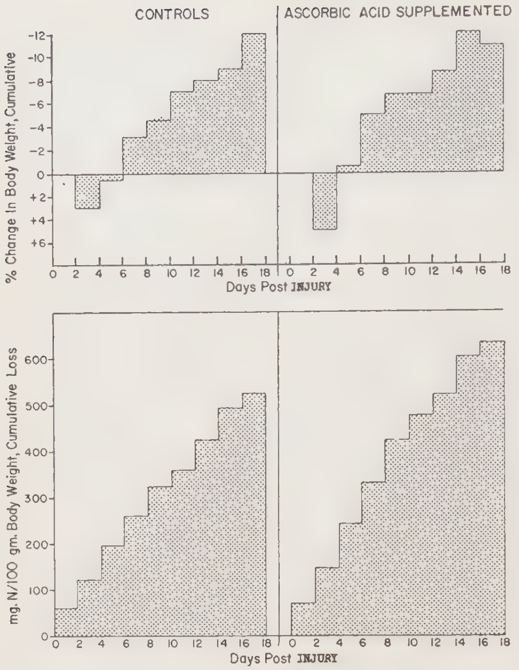

FIG. 1. Cumulative nitrogen balances and changes in body wt in thermally injured rats.

by the Fisher t test).

Prior to injury, the rats which were later to receive ascorbic acid were slightly lighter, about 5% on the average, than the controls. The rats in both groups lost about 12% of their pre-injury control weights during the 18-day period of increased urinary nitrogen excretion. About half of this weight loss can be accounted for by the observed increase in nitrogen excretion—if one assumes that the extra-urinary nitrogen was derived from tissue with approximately normal protein and water contents.

Lack of effect of ascorbic acid on the increase in urinary nitrogen excretion in this study is in apparent contradiction to the observation of Noble and Toby. However, the trauma employed in their study was leg crushing and their observations were limited to a brief (34-hour) period after injury; our observations were made in thermally injured rats and continued over a period of 18 days.

The rat, unlike man, does not require exogenous ascorbic acid for his daily normal requirements. Whether any supplemental ascorbic acid is needed by the rat following severe stress is not known. Ralli *et al.*(12) have recently studied the effect of the surgical removal of about 15% of the skin on the urinary ascorbic acid excretion by female rats. Ralli and her coworkers noted an increase in ascorbic acid excretion during the first 2 days after wounding, followed by a decrease to below the preoperative level for the next 2 weeks.

Conclusion. Loss of weight and increase in urinary nitrogen excretion, total and amino, by rats with severe thermal injury were unaffected by large doses of ascorbic acid begun shortly after injury and continued throughout the period of negative nitrogen balance (18 days). That these findings may be applicable to those species (man, monkey, and guinea pig) which depend on exogenous ascorbic acid for their normal requirements, unlike the rat, is suggested by the observations that marked increases in urinary nitrogen excretion following severe injury occur in patients receiving up to one g of vit. C daily(13).

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Site of Action of Magnesium in Treatment of Some Toxic Effects Produced by TEPP.* (22079)

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Major pharmacological actions of magnesium have been discussed in recent reviews, Engbaek(1); Smith, Winkler and Hoff(2). Various peripheral and central effects of the ion have been described, but, as noted by Engbaek(1), the question of relative sensitivities of central and peripheral structures to magnesium has not been settled.

It has been reported, McNamara, Koelle and Gilman(3), that magnesium sulfate, when used adjunctively with pentobarbital and atropine, is of value in treating poisoning produced by diisopropylfluorophosphate (DFP). The current investigation is an attempt to elucidate the site of action of magnesium in the treatment of certain effects produced by tetraethylpyrophosphate (TEPP).

Methods. Three types of experiments were performed. 1) Cats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), atropinized (1 mg/kg, i.m.) and respired artificially. Sciatic and femoral nerves of one leg were sectioned, and identical bi-polar platinum electrodes were inserted into the gastrocnemius muscles of this leg and of the opposite intact leg. Electrical activity of these muscles was recorded with a Grass electroencephalograph. In some of these experiments, electroencephalograms were recorded from screw electrodes placed in the skull. Recordings were made immediately prior to, and periodically after, poisoning with tetraethylpyrophosphate (TEPP) by injection of 0.3 to 2.0 mg/kg into the antibrachial vein. When skeletal muscle fasciculations had been produced by TEPP, magnesium sulfate or magnesium chloride (30-100 mg/kg in 5% solution) was injected intravenously. In a few experiments, after fasciculation had been abolished by the magnesium salt, the threshold for indirect stimulation of the gastrocnemius muscle of the intact leg was compared with that at the beginning of the experiments. 2. The sciatic nerve-gastrocnemius muscle technic of Wolff and Cattell (4) was used to study the response of muscle to supramaximal excitation of its motor nerve following intravenous doses of 30-130 mg/kg of magnesium sulfate in cats. 3) Rabbits were paralyzed by Intocostrin (1-2 units/kg i.v.) and were respired artificially. Dibutoline (0.5 mg/kg, i.m.) was administered because it is the authors' experience that this drug minimizes bronchial and cardiovascular effects of TEPP without preventing appearance of the "grand mal" type of electroencephalogram produced by the latter com-

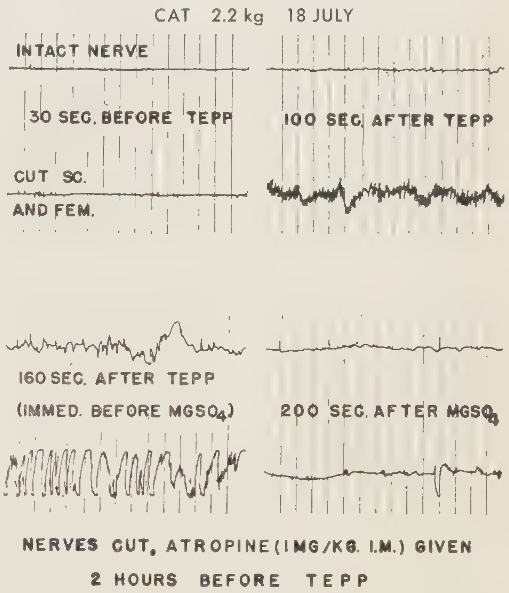

FIG. 1. Effect of magnesium sulfate (30 mg/kg i.v.) on fasciculation induced by TEPP (1 mg/kg i.v.) in cat gastrocnemius with intact innervation (upper traces) and with denervation approximately 2 hr before experiment (lower traces). Cat received atropine (1 mg/kg i.v.) about 2 hr before experiment, and given artificial respiration as necessary after poisoning. Tracings shown are similar to those obtained from 9 other cats treated similarly.

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* Abstract of this work appears in *Fed. Proc.*, 1955, v14, 373.

TABLE I. Effect of Magnesium on Response of Gastrocnemius Muscle to Supramaximal Electrical Stimulation of Its Motor Nerve.

Cat No.	Dose $MgSO_4$ (mg/kg, i.v.)	Effect on contraction height
1	30 100 (Immed.)*	No significant effect About 70% reduction
2	30 100 (immed.)	No significant effect Complete block
3	50 50 (Immed.)	No significant effect About 85% reduction
4	50 50 (Immed.)	No significant effect About 50% reduction
5	50 50 (Immed.)	No significant effect Complete block
6	60	No significant effect
7	60	About 30% reduction
8	120	" 85% "

* Immed. = Immediately after 1st dose.

pound. A screw electrode was placed in the right frontal area of the skull, potential differences between this point and an indifferent skin area being recorded. TEPP was then administered. When increased electrical activity of the brain resulted, magnesium sulfate (30-150 mg/kg, i.v.) was administered in an effort to counteract the central effects of TEPP. Atropine (1-3 mg/kg, i.v.) was injected thereafter.

Results. Group I. Fig. 1 shows clearly that the site of action of magnesium in counteracting these fasciculations is peripheral because magnesium sulfate, in a dose of 30 mg/kg i.v., quickly abolished the fasciculatory response in both the decentralized leg and the leg with intact nerves. In other experiments, doses of up to 100 mg/kg of magnesium salt were needed to abolish fasciculation. In 4 of 5 experiments, it was noted that the threshold for indirect stimulation of the muscle following abolition of fasciculation by low doses of magnesium was similar to that of the prepoisoning control.

Group II. Table I illustrates the effect of magnesium sulfate on the response of the gastrocnemius muscle to supramaximal electrical stimulation of the sciatic nerve. Doses of magnesium (30 mg/kg, i.v.) which frequently abolished fasciculations in other experiments did not alter significantly the response of the muscle to motor nerve excitation. Large doses of the magnesium ion, however, reduced the muscles' contraction height.

Group III. Magnesium sulfate in doses as high as 150 mg/kg, i.v., did not abolish the abnormal brain activity in any of 6 rabbits. When atropine sulfate was then given (1-3 mg/kg, i.v.), the "grand mal" pattern was quickly abolished. Fig. 2 illustrates these results.

Discussion. The results of this investigation show that the site of action of magnesium sulfate in the treatment of some effects of TEPP intoxication is primarily peripheral. The magnesium salt was effective in abolishing fasciculations in both the leg with sectioned nerves and in that with intact innervation. The exact mechanism of this action has not been elucidated by the current experiments, but evidence has been obtained that the magnesium effect does not involve necessarily depression of neuromuscular transmission or of repetitive cerebral or spinal cord activity. The action probably is localised, therefore, to the skeletal muscle fiber.

It has been reported that magnesium reduces the output of acetylcholine from cholinergic nerve endings, Hutter and Kostial(5). Others, Del Castillo and Engbaek(6), have demonstrated that, although the main effect of magnesium on neuromuscular function is a diminution of the amount of acetylcholine released by the motor nerve endings, the magnesium ion raises the electrical threshold of the muscle membrane and also has a small depressant effect on the acetylcholine sensitivity of the end-plates. Both of these mechanisms may well play a part in the effect on the muscle fiber made to fasciculate by TEPP.

Summary. 1. The primary site of action of magnesium in the treatment of skeletal muscle fasciculations produced during TEPP intoxication is peripheral. 2. Magnesuim apparently has little, if any, value in the treatment of the central effects produced by this poison.

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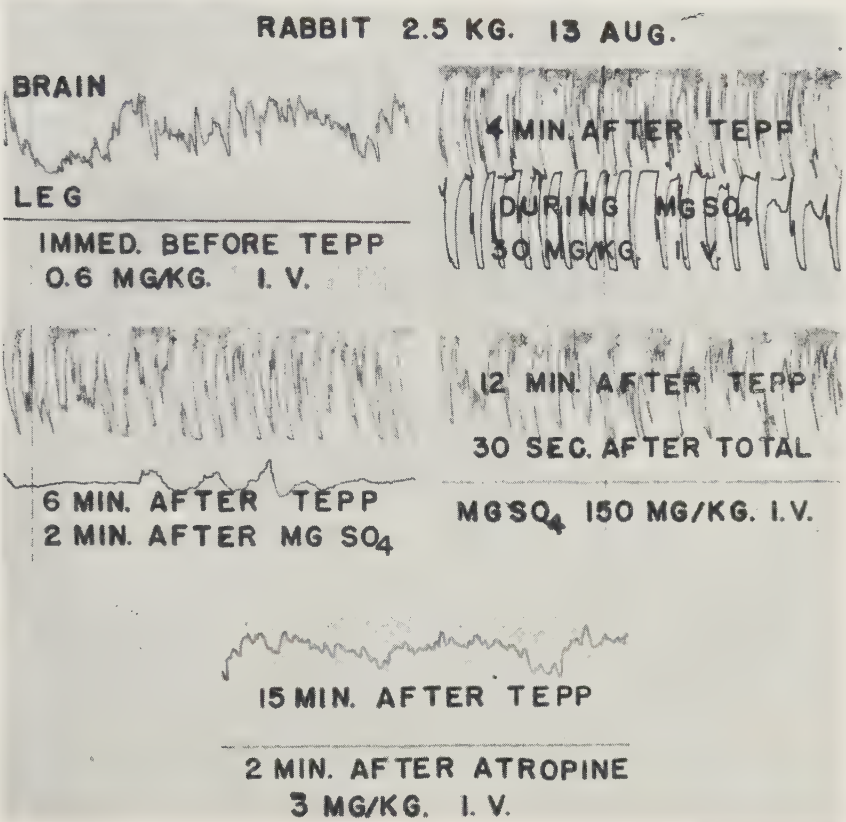

FIG. 2. Comparative effectiveness of magnesium sulfate (30 mg/kg i.v. and 7.5 min. later 120 mg/kg i.v.) on EEG hyperactivity (upper traces) and on fasciculation of gastrocnemius (lower traces) induced by TEPP (0.6 mg/kg i.v.) and comparative effectiveness of magnesium sulfate and of atropine sulfate (3 mg/kg) on EEG hyperactivity (last two panels). Rabbit received Dibutoline (0.5 mg/kg i.m.) and Intocostrin (2 units/kg i.v.) before start of experiment, and respired artificially throughout experiment.

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Presence of ACTH in Pituitary Gland of Pacific Salmon (*O. keta*). (22080)

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The existence of a relationship between the pituitary gland and interrenal tissue of fish (*Astyanax mexicanus*) has been reported by Rasquin(1). Following injections of ACTH of mammalian origin, the anterior interrenal tissue, which is considered homologous with the mammalian adrenal cortex, responds by hypertrophy, hyperplasia, and increased vascularization. Hatay(2-4), and Fontaine and Hatay(5-7) show that the corresponding tissue in the eel (*Anguilla anguilla* L) is rich in ascorbic acid and that following hypophysectomy, this component is decreased and the interrenal tissue has become atrophic. In contrast, Pickford(8) was unable to detect changes in the cortical tissue of *Fundulus* following hypophysectomy.

It seemed of interest to determine if a corticotropic substance exists in the teleost pituitary, which like mammalian ACTH, can deplete adrenal ascorbic acid in the hypophysectomized rat. Assays of lyophilized pituitary glands* and of certain pituitary fractions of a species of Pacific salmon (*Oncorhynchus keta*), which will be reported, show clearly the presence of ascorbic acid-depleting activity.

Materials and methods. Preparation of extracts. Pituitary glands from freshly caught salmon were preserved in solid carbon dioxide within 3 hours of sacrifice. Following lyophilization, the dried tissue was ground with mortar and pestle. Immediately before use a weighed amount of tissue was extracted in an appropriate volume of N/1000 hydrochloric acid. This preparation was injected without further processing. *Preparation of fractions.* Fraction #1. Lyophilized pituitary tissue was extracted at room temperature for 24 hours in a solution of glacial acetic acid and acetone;† one g of tissue in 16 ml of acid and 2.5 ml acetone. One volume of distilled water was then added. After thorough mixing the suspension was centrifuged, the insolubles being preserved by lyophilization for further study. To the supernatant acetone was added, with stirring, until heavy turbidity was evident. At this point additional acetone was added cautiously until a flocculent precipitate formed, leaving a clear supernatant. The solids were collected by centrifuging, and washed twice with ethyl ether. The washed precipitate was redissolved in water, enough glacial acetic acid being added to assure solution. After warming at 37°C for a few minutes to remove excess ether, the solution was frozen and lyophilized. Yield: approximately 13% of starting weight. Fraction #2. To the supernatant fluid remaining after the precipitation of Fraction #1 was added at least one volume of acetone. If necessary,

TABLE I. Adrenal Ascorbic Acid Depletion in Hypophysectomized Rats following the Intravenous Administration of Various Pituitary Preparations of the Pacific Salmon *(O. keta)*.

Preparation used	Dose* (μg)	No. of animals	Adrenal ascorbic acid depletion (mg %)
Lyophilized tissue	25	3	73, 68, 105
	100	3	101, 177, 119
Fraction #1	50	2	222, 188
	100	3	214, 167, 182
Fraction #2	50	3	137, 105, 150
	100	3	149, 156, 70
Fraction #1	10	3	83, 138, 97
	20	2	124, 96
	40	4	176, 143, 195, 162
Fraction #2	12.5	3	37, 79, 62
	25.0	3	72, 64, 84
	50.0	3	159, 134, 177

* Dose based on 100 g of body wt. Left adrenals removed immediately before intravenous administration of test dose. Right adrenals removed 1 hr following test dose.

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* Pituitary glands were kindly supplied by Mr. Roger Burrows and his colleagues of the Fish and Wildlife Service, U. S. Department of Interior, Entiat, Wash.

† This procedure is a modification of the method reported by Payne, Raben and Astwood(9) for extraction of hog pituitary glands.

TABLE II. Adrenal Ascorbic Acid Depletion Assays in Hypophysectomized Rats Injected with Pituitary Preparations of the Pacific Salmon *(O. keta)* and with U.S.P. Standard Corticotropin.

No. of animals	Test material	Test dose per 100 g body wt	Mean response, adrenal ascorbic acid depletion	Assay data
8	LSP*	25 μg	110	$S_c = \pm 20$†
8	"	50 "	131	$b_c = 86.7$
8	"	100 "	160	$\lambda_c = .23$
7	USP Std	.5 mU§	103	$R\frac{(USP)}{(LSP)} = 48.9$‡
7	"	1.0 "	134	95% limits 35.7-66.8
7	"	2.0 "	158	
3	Fraction #1	25 μg	109	$S_c = \pm 32$
4	"	50 "	168	$b_c = 143$
3	USP Std	.5 mU	93	$\lambda_c = .22$
3	"	1.0 "	120	$R\frac{(USP)}{(Fr.1)} = 29.4$
3	Fraction #2	25 μg	85	
3	"	50 "	95	$S_c = \pm 24$
3	"	100 "	130	$b_c = 84.3$
3	USP Std	.5 mU	93	$\lambda_c = .29$
3	"	1.0 "	120	$R\frac{(USP)}{(Fr.2)} = 52.8$
3	"	2.0 "	149	

Assays conducted according to the method of Sayers *et al.* Left adrenals removed immediately before injection of test dose. Right adrenals removed 1 hr following test dose.

* LSP = Lyophilized salmon pituitary tissue.

† S = Stand. dev.; b = slope; λ = s/b; subscript 'c' for combined data.

‡ R = Ratio of potency.

§ mU = Milliunits.

further acetone was added to reduce the turbid suspension to a flocculent precipitate. The solids were collected by centrifuging and prepared for lyophilization as with Fraction #1. Yield: approximately 11% of starting weight. *Assays.* Adrenal ascorbic acid depletion assays were conducted according to the method of Sayers, Sayers, and Woodbury (10). Male Sprague-Dawley rats, 125-150 g body weight, were hypophysectomized 24 hours prior to assay. Test preparations dissolved in N/1000 hydrochloric acid, were injected, after laparotomy, via the vena cava. Preliminary tests were made to determine whether ACTH activity was present. The results of these were used to estimate dose levels in assays against the USP standard corticotropin.

Results. Data obtained in preliminary tests are presented in Table I. It is apparent that, in these preparations from salmon pituitary tissue, a physiological activity is present which is qualitatively the same as that observed in pituitary preparations of mammalian origin. Potency assays against the USP standard corticotropin are summarized in Table II. The results indicate an ACTH content in the lyophilized tissue of about 20 milliunits per mg. Because of difficulties in field conditions under which these glands were collected, freezing in solid carbon dioxide was delayed 2 to 3 hours after removal. This lapse of time may have resulted in loss of physiological activity. Hence it is possible that freshly frozen glands would show a greater ACTH content.

Assay data on Fractions #1 and #2 indicate some concentration of ACTH activity in the former. On a weight basis Fraction #2 appears about as active as the starting material.

Discussion. The findings reported establish the presence of ACTH activity in the teleost pituitary. A recent report by Nelson, O'Connell, and Haines(11) describes the presence of ACTH activity in sardine fish meal. The results described here may account in part for this finding, if small quantities of pituitary tissue were present in the meal.

The presence in the teleost of a pituitary component capable of producing adrenal cortical stimulation in the mammal suggests that the protein or peptide structure responsible for this activity was of metabolic importance early in the evolutionary development of the species. Whether or not the role of ACTH in the phenomena associated with response to stress in mammals is the same in fish remains to be determined.

Summary. Lyophilized pituitary tissue of the Pacific salmon (*O. keta*) and pituitary fractions, were examined for ACTH activity in hypophysectomized rats. The results indicate the presence of a component in the teleost pituitary gland capable of depleting the stores of adrenal ascorbic acid in the rat. In this respect the preparations are qualitatively identical with corticotropins (ACTH) of mammalian origin.

The authors wish to acknowledge with gratitude the interest in this work of Dr. O. H. Robertson, Stanford University, who first introduced them to the endocrine problem in fish.

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Rate of Glycogenesis in the Liver of the Rat After Parenteral Administration of Glucose, Fructose and Invert Sugar.* (22081)

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It is generally considered that fructose forms glycogen in the liver more rapidly than glucose. This assumption is based upon *in vitro* experiments(1) and studies in which the two sugars were given to rats by mouth (2). Since in clinical practice sugars are administered intravenously for supplying calories to patients unable to take food by mouth, it was considered of interest to study the rate of glycogenesis in the liver when glucose, fructose, and invert sugar are given parenterally.

Method. Normal white rats, weighing 200 to 300 g, were fasted for exactly 24 hours, water being allowed *ad lib.* Sugar, in 10% solution, was injected intraperitoneally. A dosage of 2 g per kg of body weight was used. This dosage was selected because it was found by experiment to give a suitable load to the animal and it is below the level at which utilization might be diminished by biological limitations. A carefully measured quantity of sugar solution was injected intraperitoneally into the fasted rat. At different intervals after administration, animals were anaesthetized with nembutal and the whole liver was removed for analysis. Liver glycogen was determined by an anthrone method developed in our laboratory(3). Our procedure was to give sugar solution to 6 rats and the same volume of physiological saline to 2 control animals. Liver glycogen determinations were then carried out upon the group of 8 animals. Groups of animals were sacrificed at approximately hourly intervals for periods

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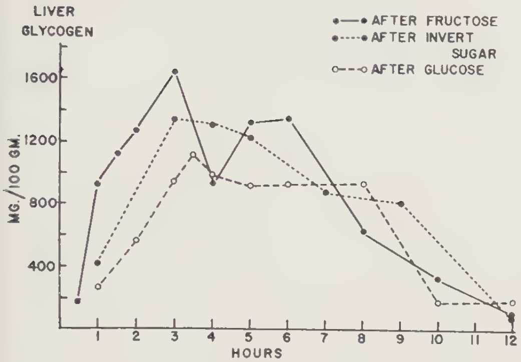

FIG. 1. Rate of glycogenesis in liver in fasted rats after intraperitoneal injection of fructose, invert sugar and glucose. Dosage was 2 g/kg of body wt.

extending up to 12 hours after administration. Observations were made following the administration of glucose, fructose and invert sugar. Comparative results were thus obtained for the 3 sugars using identical experimental procedures.

Results are shown in Fig. 1. Liver glycogen formation after fructose injection was approximately twice as fast as after glucose injection for 3 hours; at the fourth hour the curve after fructose administration dropped sharply but returned to a higher level than the glucose and invert sugar curves at the 5 and 6-hour points and then sloped off at about the same level as for the other two sugars between 7 and 12 hours after administration. The curve showing liver glycogen content after glucose is the lowest level observed for the 3 sugars. The curve showing liver glycogen values after invert sugar injection has a level about midway between the curves for fructose and glucose for 6 hours after administration and then occupies a level about the same as the other 2 sugars until the twelfth hour. The liver glycogen content 12 hours after administration returned to the pre-injection level for each sugar.

At the highest point of the glycogen curve for each sugar the per cent of the administered dose that had been converted into glycogen was 28.8 for fructose, 20.3 for invert sugar and 18.1 for glucose.

The intermediate position of the invert sugar curve is as would be expected of this sugar, since it is an equimolar mixture of glucose and fructose. The sharp dip in the curve after fructose at the 4-hour period is difficult to explain. The blood glucose after fructose injection had reached its highest level, 120 mg%, at the 5-hour point. Since fructose gives rise to increased blood glucose, possibly the effect of the latter caused the upturn of the glycogen curve at this point after fructose administration. This suggestion is supported by the fact that the curve after invert sugar maintained a steady level at the 4-hour point which shows the influence of glucose at this time.

Summary. Rate of glycogenesis in the liver of the rat after intraperitoneal injection of sugar solution was found to be highest after fructose, lowest after glucose, and intermediate between fructose and glucose after invert sugar. During the first 3 hours after injection the rate of glycogen formation was approximately twice as fast after fructose as after glucose, a finding that explains, in part, the more rapid disappearance from the blood of fructose than glucose after parenteral administration.

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Implantation of Functional Erythropoietic Elements Following Total-Body Irradiation.* (22082)

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(Introduced by A. Hollaender.)

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It has been demonstrated that recovery from lethal doses of X irradiation is promoted by the injection of living, nonirradiated bone marrow(1). It has been postulated that the introduced marrow cells proliferate and are responsible for recovery by cellular repopulation, or that they elaborate some humoral substance which stimulates recovery of the indigenous marrow. Transitory activity of the injected cells pending recovery of the irradiated marrow has also been suggested as the beneficial role played by the transfused marrow. It seems reasonable that marrow cells injected into an irradiated animal might proliferate and contribute cellular elements to the peripheral circulation of the host because of (a) the inhibiting effects of total-body irradiation on the immune response (2,3), (b) the demonstration of homologous transplantation into embyronic hosts(4-6) and of the postpartum tolerance by these hosts of skin transplants from the donor (7-10), and (c) the acquired tolerance by irradiated marrow-injected mice of skin grafts from the marrow donor(11). Demonstration of the establishment of a functional implant of foreign bone marrow in an irradiated animal would indicate that the amount of genetic difference which can be tolerated between the donor of a tissue transplant and the recipient may be increased by irradiation of the recipient, and would lend support to the cellular repopulation hypothesis as a factor in radiation recovery. Experiments with rats are described, in which marrow carrying an immunogenetic marker is utilized. It is shown that implanted marrow cells do contribute erythrocytes to the peripheral blood of irradiated animals for extended periods of time.

TABLE I. Schedule of Treatment of Rats Injected with Immunogenetically-Labeled Marrow.

Exp. No.	No. of animals	Dose (r)	Type of host	Type of implant	Time of marrow inj. post-irrad. (hr)
1a	10	900	C or D	CD	0– 3
b	10	900	CD	—	—
c	10	0	C or D	CD	0– 3
2a	11	750	C or D	CD	18–24
b	7	750	CD	—	—
3a	21	750	C or D	D or C	18–24
b	11	750	CD	—	—
c	7	0	C or D	D or C	18–24

Experimental. Cellular antigens C and D in the rat are controlled by a single pair of alleles; erythrocytes of the 2 homozygotes, C/C and D/D, carry antigens C and D respectively, and those of the heterozygote, C/D, carry both antigens C and D(12). Cells of either homozygous type may be detected in a mixture of both by the specific agglutination of each with its homologous antiserum. Animals segregating for these genes as well as specific reagents against each antigen were kindly supplied by Dr. Ray D. Owen of the California Institute of Technology. Rats of one antigenic type were irradiated and subsequently injected with bone marrow of another type; the irradiation and injection schedules used in these experiments are described in Table I. Marrow was forced from the femora of freshly decapitated rats, suspended in Tyrode's solution, and immediately injected into the jugular vein of the anesthetized recipient. In the first experiment, the marrow from one femur was injected into each recipient, whereas marrow from two femora was generally used in Experiments 2 and 3. Unirradiated controls were similarly injected with the carrier. At various intervals following administration of the bone marrow, a drop of blood was collected from the tail of each surviving marrow-injected animal and suspended in citrate.

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* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

TABLE II. Results of Bone Marrow Administration following Total-Body Irradiation. Each day's results are represented by a fraction; numerator represents No. of animals showing in peripheral blood erythrocytes of implanted antigenic type, and denominator represents No. of surviving animals; on days when no blood typing was done, no numerator is present.

Exp. No.	Treatment	Time after irradiation (days)										
		0	6	8	10	14	18	20	22	28	35	46
1 a	X-ray + marrow	–/10	0/ 5	–/ 3	–/ 3	1/ 1	1/ 1	–/ 0				
b	X-ray + Tyrode's	–/10	–/ 8	–/ 0								
c	Marrow	–/10	–/10	–/10	–/10	0/10						
2 a	X-ray + marrow	–/11	–/11	0/ 4	–/ 4	3/ 3	3/ 3	3/ 3	2/ 2	2/ 2	2/ 2	1/1
b	X-ray + Tyrode's	–/ 7	–/ 7	–/ 6	–/ 6	–/ 5	–/ 5	–/ 5	–/ 4	–/ 4	–/ 3	–/3
3 a	X-ray + marrow	–/21	–/21	–/19	0/17	11/16	–/16	12/14	–/14	12/14	12/14	
b	X-ray + Tyrode's	–/11	–/11	–/10	–/ 8	–/ 5	–/ 5	–/ 5	–/ 4	–/ 4	–/ 4	
c	Marrow	–/ 7	–/ 7	–/ 7	0/ 7	0/ 7	–/ 7	0/ 7	–/ 7	0/ 7	0/ 7	

Each sample was washed once in 100 volumes of saline and diluted to a 2% cell suspension; this cell suspension was tested against anti-implant-type serum, against saline, and in some instances against antihost-type serum. Antisera were used in concentrations sufficient to effect complete agglutination of a 2% suspension of homologous erythrocytes. The mixtures were lightly centrifuged and examined for evidence of agglutination upon resuspension of the sedimented cells. The resuspended mixture was often examined microscopically to detect cell aggregates too small to discern with the unaided eye.

The *results* of the experiments are summarized in Table II. By the end of the second week, erythrocytes from the implant could be demonstrated circulating in the peripheral blood of most of the surviving irradiated, marrow-injected animals; the injected but unirradiated animals, on the other hand, showed no evidence of implanted cells at any time. The first indications of erythrocytes from the implant were generally visible only microscopically. A positive microscopic agglutination was usually followed within a few days by positive macroscopic agglutination. The length of time a marrow implant will persist is unknown; at the time of this communication, one mosaic animal from Exp. 2 is still living after 147 days, and 12 animals from Exp. 3 are still alive after 95 days with no evidence of implant regression.

The methods described by Wilkie and Becker(13) for quantitative determination of hemagglutinin titers were modified slightly and used to estimate the composition of the erythrocyte populations of the surviving rats in these experiments. First estimates on the thirty-seventh day postirradiation on 3 animals from Exp. 3 showed about 85% implant- and 15% host-type cells in two animals, and less than 5% implant- and 90% host-type cells in one animal. On the 65th and 95th days, postirradiation values for 12 animals ranged from less than 10% implant-type cells to close to 90%, the majority of the animals showing more than 60% implant-type cells. The sum of independent estimates of the percentage of host-type and implant-type cells was close to 100%, and agreement between estimates made on the 37th, 65th and 95th days was excellent. The sole survivor of Exp. 2 showed about 95% implant-type and 5% host-type cells on days 91, 112 and 147 postirradiation.

Discussion. The factors which determine the fate of tissue transplants have long been of great interest. It is known that the compatibility of a transplant is a function of the genetic similarity between the donor and the recipient; transplantation between identical twins or between individuals of a highly inbred strain is usually successful, whereas transplantation between less closely related individuals of the same species or between species is generally unsuccessful. There are, however, conditions under which successful transplantation can occur in the presence of greater genetic diversity between recipient and donor. If an embryonic animal is confronted with a foreign antigen throughout the development of its antibody-producing system, the postpartum reactions of the animal to the same antigen are profoundly affected. If the antigen is cellular, the foreign cells may continue to divide and may actually differentiate and function in the host animal; this has been demonstrated for naturally administered erythroid elements in dizygotic twins in cattle(4,5) and in man(6) and for artificially administered erythroid elements in the rat [Ripley and Owen in(14)]. In all cases, the administered cells become established and produce mature erythrocytes giving rise to persistent erythrocyte mosaicism. Anderson *et al.*(10) have shown in cattle that mosaicism of one animal for erythrocytes of his twin renders that animal tolerant of skin grafts from the twin. Billingham *et al.*(8,9) have further shown that mice and chickens injected as embryos with homologous cells are more tolerant as adults of skin grafts from the donor of the injected cells.

It is probable that the reaction of an irradiated rat to a foreign cellular antigen is analogous to the reaction of an embryonic animal to the same cellular antigen. It has been shown by Dixon *et al.*(2) that the immune response of the rabbit can be temporarily eliminated by total-body irradiation in the dose range used in the experiments reported in this communication. It might be postulated that postirradiation administration of the cellular antigen results in continuous exposure of the reforming antibody-producing system to the foreign antigen; consequently, the reformed system is tolerant of a foreign antigen to which the original was not tolerant. Indeed, Main and Prehn(11) have shown that irradiated mice of one strain injected with bone marrow of a second strain are subsequently tolerant of skin grafts from the second strain; this observation, they conclude, is consistent with the hypothesis that the injected marrow elements proliferate.

Demonstration of persistent erythrocyte mosaicism in which (in some cases) more than 80% of the erythrocytes in the peripheral circulation have been derived from the implanted marrow makes it unnecessary to postulate a humoral factor or a transitory activity of the implant as the mechanism responsible for recovery of the erythropoietic and inferentially myelopoietic potential in irradiated rats treated with homologous marrow. The possibility remains that the introduced cells may produce, in irradiated animals, some substance which stimulates recovery and proliferation of marrow. It is recognized that an important contribution of erythropoietic recovery to general radiation recovery is not demonstrated in the data reported here, since no recovery-promoting effects of the marrow are evident (Table II). This observation is consistent with what is known of radiation death in rats, where the contribution of intestinal damage outweighs and precedes that of marrow damage(15).

An explanation tentatively hypothesized by Cole(16) to explain the radiation recovery brought about by the administration of living cells is that the injected cells repair genetic damage incurred by the irradiated cells by a process similar to bacterial transformation or transduction; this hypothesis is to explain his finding that nuclei and deoxyribonucleic acid extracts of spleen cells have recovery-promoting activity. On this hypothesis, it might be suggested that in the present case the cells of the implant antigenic type are descendants of a selected subpopulation of irradiated cells in which the damage has been repaired by transformation. Bacterial transformation occurs independently for unlinked loci(17,18). Complete transformation of a diploid cell from one homozygous type to another would consequently involve the intermediate formation of the heterozygous type, *i.e.*, CC→CD →DD, which was not detected. That the estimated proportions of CC and DD cells account for all erythrocytes is clearly incompatible with the concept of transformation at the chromosomal level.

Summary. Rats have been irradiated with doses of X-rays in the LD_{50} range and subsequently injected with homologous bone marrow carrying an immunogenetic marker. Erythrocytes carrying the label can be detected in the peripheral circulation of these animals within 2 to 3 weeks after administration of the marrow; unirradiated but marrow-injected controls do not show erythrocytes from the implant. Marrow implants have persisted and functioned for as long as 147 days at the time of this communication.

The implanted marrow contributes as much as 80% of the peripheral erythrocytes.

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A Simple Circulatory Bypass System.* (22083)

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Some method of total circulatory bypass is necessary to accomplish open intracardiac surgery. Ideally such a system should entail a minimum of danger to the subjects accompanied by a maximum of simplicity of working parts and operability. The use of homologous and heterologous lungs in combination with some type of mechanical pump has been shown by others(1,2) to most closely approach this ideal. This is a report of experimental work with an homologous lung system which has been further simplified.

Methods. Mongrel donor dogs weighing 10 to 20 kg were bled to death after receiving one mg of heparin per kilo of body weight. Heparin was then added to this blood in amounts of one mg per 100 cc of blood. This blood was used to prime the extracorporeal system, to replace blood loss, and to maintain the operated dog's blood pressure during surgery. No blood was given to any of the dogs after completion of the experimental surgical procedure. Following exsanguination of the donor dog, the left lung was removed and the bronchus and pulmonary artery were cannulated, the former being connected to a respirator and the latter to the discharge end of a 300 cc graduated cylinder. Experimental dogs weighing from 9 to 20 kg were then anesthetized with morphine and nembutal and the

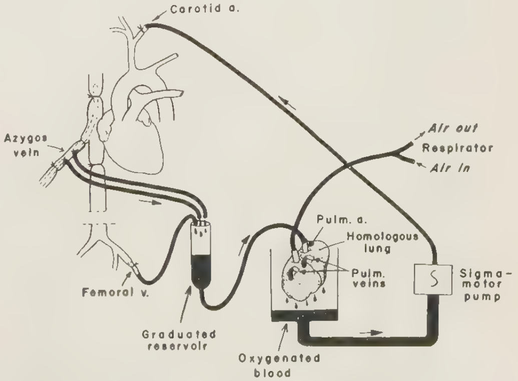

FIG. 1. Cannula connections of homologous lung blood oxygenator.

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* This work was supported by funds obtained from the Life Insurance Medical Research Fund.

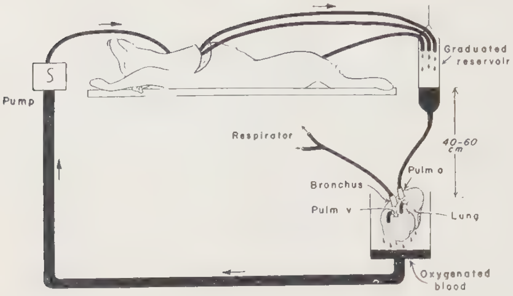

FIG. 2. General arrangement of homologous lung blood oxygenator.

femoral artery and vein cannulated and connected to an electronic recording manometer. Cannulae were also placed in the other femoral vein, the distal portion of the azygos vein, and the superior vena cava via the proximal portion of the azygos vein. These cannulae were all led to the intake portion of the 300 cc graduated reservoir. Another cannula was placed in the proximal portion of the dog's carotid artery and led through a Sigmamotor pump to a bottle collecting the oxygenated blood. All cannulae were filled with a saline solution containing one mg of heparin per kilo of body weight of the dog. The details of the various cannula connections are shown in Fig. 1 and the general arrangement of the lung and pump is shown in Fig. 2. During operation of the extracorporeal system, venous blood is drained into the graduated cylinder, which can be used as a flowmeter, after equilibration of arterial and venous flows. Venous blood is then forced by gravity through the artificially respirated lung, pressures of 40 to 60 cm of water (blood) being more than adequate for flows needed in these experiments. Oxygenated blood then flows out of the pulmonary veins, is collected in an inverted bottle, and pumped back to the dog with a Sigmamotor pump of variable speed. With the circulatory bypass system in operation as described, 12 experimental ventriculotomies have been done, under clean but not sterile conditions, during a period of 30 minutes of complete bypass of the heart and lungs, this period being preceded and followed by 2- to 3-minute periods of partial bypass. During the ventriculotomies the right ventricle was kept open for a period of 5 minutes. Continuous recordings were made of the arterial and venous pressures and usually 2 measurements were made of the blood flow. Low blood flows were used according to the azygos flow principles determined by others(3). After the experimental animal was disconnected from the circulatory bypass system the heparin in its blood was neutralized by the addition of protamine sulfate. The amount of protamine to be given was determined by dividing the total amount of heparin used by 2 and giving one cc of 1% protamine sulfate for every 10 mg of heparin. This was diluted in 100 cc of 5% glucose and given by intravenous drip over a 15- to 20-minute period. After administration of the protamine, titration tests were done to determine if sufficient quantity had been given.

Results. Twelve experimental animals have been subjected to this procedure. The first 5 animals survived the experiment without sequelae. The next 5 animals all died in the immediate postoperative period, severe bleeding being the cause of death. Despite the fact that adequate protamine had been given these dogs to neutralize the heparin by *in vitro* testing, their blood did not clot *in vivo*. In attempting to discover the cause of this reduced coagulability, it was discovered that the glass and plastic equipment that was being reused in successive experiments was not being adequately cleaned. All of this equipment was then either replaced or cleaned thoroughly with acid solution. The 2 dogs that have undergone the experiment since then have sur-

TABLE I. Blood Flow during Homologous Lung Experiments in 12 Dogs.

Survival	Flow (cc/kg/min.)	Flow (cc/min.)	Remarks
+	44–58	540–720	Aborted 1st day postop.
+	52	540	
+	36–40	540–600	
+	20–25	240–300	
+	44	600	
0	39	420	
0	44	600	
0	27	480	
0	40	420	
0	25–29	240–280	
+	33	420	
+	31–35	420–480	Ventricular fibrillation

vived without sequelae and have shown no evidence of bleeding tendency.

Summary. The use of an homologous lung is at present the simplest and most efficient method of providing extracorporeal oxygenation of blood. A simplified method of using such a lung experimentally has been described. In this experimental study of 12 animals there were 7 survivors, the 5 fatalities being caused by severe bleeding resulting from contaminated equipment.

The authors wish to express their appreciation to Mr. Dusan Surman, for his technical assistance.

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Relationship of Salivary Tryptophane to Lactobacillus Colony Morphology and Dental Caries.* (22084)

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In a previous investigation of the salivary microflora of dental caries-free and caries-susceptible humans, an association of smooth colony morphology of lactobacilli with saliva of caries-free subjects has been described(1). Rough colony types predominated in saliva of the caries-susceptible subjects. The observation that a tryptone medium as well as tryptophane induced rough to smooth dissociation in colony morphology of oral lactobacilli prompted the present study of this action of tryptophane on lactobacillus colony morphology, and the possible relationship of salivary tryptophane to caries resistance.

Materials and methods. The lactobacilli used in this study were of human oral origin. The cultures were classified into 3 types, smooth, intermediate and rough on the basis of colony morphology on tomato agar. The rough colony type is flat, translucent, with an indented to serrated edge, and is stable on consecutive passage on tomato agar. Tomato juice agar was prepared according to the Hadley method(2). Rogosa's Lactobacillus Selective Medium(3), used for growth and fermentation studies, was modified for general use as a liquid medium by omission of the agar, glacial acetic acid, and 5 g of sodium acetate per liter.

To test the action of tryptophane the medium was modified to contain 10 g acid hydrolyzed Casamino Acids (Difco) instead of trypticase; 0.013 g dl-tryptophane; and 15 g agar. Additional amounts of dl-tryptophane were added as required. Modifications of the Ehrlich (paradimethylaminobenzaldehyde) and Hopkins-Cole (glyoxylic acid) tests were studied for determination of tryptophane in saliva, and the latter test was selected. The procedure has been described by Block(4), with the essential modification that the concentration of H_2SO_4 was reduced to avoid undesirable reactions with other constituents of saliva.

Three ml of resting and 3.0 ml of stimulated saliva were collected from a subject and mixed. Two ml of the mixture were placed in a 10.0 ml volumetric flask and 0.5 ml of M/25 $CuSO_4$ and 0.5 ml of glyoxylic acid were added. The flask was held in an ice-and-water bath for 5 min., then 5.0 ml of 80% H_2SO_4 were slowly added. The flask was held in the cold bath for 10 min. then placed in boiling water for 5 min. After cooling 2 min. at room temperature the contents were mixed by rotating the flask and cooled 8 more min. The contents were diluted to 10 ml volume with 50% H_2SO_4, incubated at room temperature for 15 min., then observed in a Coleman Junior Spectrophotometer at 515 and 570 mu. The reference standard was similarly prepared except that 0.5 ml distilled water was substituted for glyoxylic acid. The characteristic absorption maxima in this test for tryptophane and indol are respectively 570 and 515 mu. The test procedure described by Block, when applied to pure solutions of tryptophane and indol, results in colored reaction mixtures that follow the Lambert-Beer Law; concentration $= -K$ log transmittance. Substitution of 80% H_2SO_4 introduced some error. The error in transmittance at a known concentration of 0.02 mg tryptophane was about 3%, at 0.10 mg about 15%. The values for time, volume, concentration and temperature are all critical. The subjects used as sources of saliva in this study were all young adults classified as caries-free or "immune," and caries susceptible on the basis of clinical and roentgenologic examinations. The susceptibles showed past or present caries, whereas the immunes showed no evidence either past or present.

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Results. The original observation was that certain rough colony strains of oral lactobacilli exhibited a previously unobserved change to smooth colony morphology when grown on tryptone glucose extract agar. This change was demonstrable regularly on this medium, and was reversible after 2 or 3 transfers on tomato agar. The addition of 0.5% tryptone to tomato agar also induced the rough to smooth colony variation. In addition, some rough colony lactobacilli, grown on tomato agar containing 0.004% dl-tryptophane, underwent rough to smooth dissociation which did not occur on this medium without added tryptophane. However, 10 rough colony lactobacillus strains cultured on modified Rogosa's lactobacillus selective medium to which 0.0013% dl tryptophane had been added, at pH 5 and 7 showed only slight changes in colony morphology as compared to the changes observed on tomato agar. The addition of 0.02% dl-tryptophane or indol to this medium caused 7 of the strains to vary to smooth morphology. Although the results were variable, colony dissociation induced by tryptophane or indol was more common at pH 5 than at pH 7. Tryptophane and indol were about equally effective and strains of lactobacilli reacted to either, both or neither.

The rough to smooth variation was seen in all colonies which developed on media with added tryptophane, and all colonies which were picked from such media were capable of reversion. Aliquots of broth cultures developed approximately equal numbers of colonies on media with and without added tryptophane. The variation was usually reversed on first passage without added tryptophane, although some strains required 3 to 4 transfers before all smooth characteristics were lost. Successive passages on tryptophane-containing media did not render the smooth variants more resistant to reversal when removed from the influence of tryptophane. Thus the effect of tryptophane was transitory, perhaps suggestive of adaptive enzyme formation. The fermentative capacity of the smooth variants for a number of carbohydrates did not differ from the parent cultures, the original acidogenic and aciduric properties being maintained. Attempts to differentiate smooth variants from rough parents by several common capsule stains, especially by use of Alcian Blue, a carbohydrate specific stain described by McKinney(5), were not successful.

Turner and Crowell(6) have reported an association of tryptophane or tryptophane-like compounds with saliva of caries-free humans. More recently, however, Stack(7) has asserted the independence of salivary tryptophane and caries experience. In view of the above findings chemical analyses of the tryptophane and indol content of caries-immune and susceptible individuals were performed.

Descriptions of the subjects whose salivas were analyzed and results of the analyses are given in Table I. The statistical analyses of the data show a significantly greater amount of both tryptophane and indol in immune salivas. The Hopkins-Cole test is not specific

TABLE I. Analyses of Salivas for Tryptophane and Indol in 31 Subjects.

Caries immune			Caries susceptible		
Solution color	% transmission at: 570 mu	515 mu	Solution color	% transmission at: 570 mu	515 mu
Yellow	100.0	100.0	Yellow	95.0	92.2
Purple	81.3	81.3	"	99.2	97.0
"	40.0	40.0	Pink-gray	71.2	81.0
"	44.0	64.3	Yellow	94.3	95.2
"	25.3	46.0	Pink	52.1	71.2
"	28.2	41.2	Pink-brown	62.0	63.1
"	38.0	41.2	"	66.3	66.2
"	50.3	63.3	Yellow	82.1	77.1
"	36.0	58.1	Purple	53.1	88.3
"	13.1	22.0	"	56.2	106.0
"	45.0	56.1	Pink	29.3	42.2
"	34.0	61.1	"	61.1	66.3
"	51.2	57.1	"	28.0	39.2
"	28.2	50.1	Yellow	29.1	40.0
"	28.3	53.2	"	66.2	52.1
			Pink	40.0	57.2

"t" Tests

Tryptophane, 570 mu: $t = 2.33$ Probability $= 0.038$
Indol, 515 mu: $t = 2.087$ Probability $= 0.05$

Chi-Square Test for Tryptophane Color

	Purple	Non-purple	Totals
Immunes	a 14	b 1	15
Susceptibles	c 2	d 14	16
	16	15	

Chi-square $= 20.26$; Probability $=$ less than 0.001

for free tryptophane alone, as combined tryptophane or compounds having similar configurations may give a color reaction. However, the deep purple color seen with most immune salivas is the same as that given by pure solutions of tryptophane.

The association of smooth colony salivary lactobacilli and high salivary tryptophane in caries immunes prompted an additional study. Several dental students having a mixed flora of salivary lactobacilli were given paraffin containing dl-tryptophane to chew intensively during 6 hours per day for one week. This procedure increased salivary tryptophane content but had no effect on the numbers of smooth and rough colony type lactobacilli isolated from their salivas.

Discussion. Dissociation of colony morphology of a culture of microorganisms due to addition of a single compound is not novel. Rogosa and Mitchell(8) have reported similar results on adding sorbitan monooleate (Tween 80) to a medium. They attributed the change in morphology to alteration of the nutritional environment. Thus, alteration of colony morphology of an entire population was not the result of genetic changes, but rather a difference in phenotypic expression of genetically similar populations in two different environments. It is rather likely that such is the case in tryptophane-induced dissociation.

The finding of significantly greater amounts of tryptophane or tryptophane-like compounds in caries-free salivas compared to caries-susceptible has confirmed the work of Turner and Crowell(6), and seems to strengthen their hypothesis that salivary tryptophane levels are related to the presence or absence of caries. It is not, however, necessarily at variance with the results of Stack (7), since a high salivary tryptophane content was found in one individual (age 14) with clinically rampant caries. In addition, Stack's conclusions might be confirmed by examining selected items of data from Table I, since his study apparently included only 3 caries-free individuals. Comparison of the results with the 2 clinically different populations in the present work, and in that of

Turner and Crowell(6), leads to other conclusions. It is also well to bear in mind that factors which may be correlated with caries immunity may be unrelated to susceptibility.

Obviously, the relationship of high salivary tryptophane or indol and the caries-immune state is not one of cause and effect. Furthermore, the rough to smooth colony dissociation of lactobacilli produced by tryptophane or indol *in vitro* is different both in extent and kind from the similar action of immune saliva(1). The latter is effective on most cultures of lactobacilli and is definitely affected by pH. It appears to be a selection process associated with inhibition of growth and results in organisms with modified physiological activities. As noted above, none of these apply to the tryptophane-indol induced variants. Likewise the low count of predominantly smooth colony lactobacilli in caries-immunes suggests that the above action of immune saliva occurs in the oral cavity, while the present findings indicate that high salivary tryptophane is not present in all immunes and does not affect the number of rough colony lactobacilli in susceptible mouths even when the salivary concentration of tryptophane is increased.

It is equally clear, however, that caries immunity and high salivary tryptophane are probably related in some way when both are present. In such cases, even the rough to smooth dissociation in lactobacillus colonies may be related to tryptophane present in immune saliva. In cases of low salivary tryptophane in immunes, this type of resistance t caries may not be involved.

Summary. 1. Addition of dl-tryptophan or indol to media induces a rough to smoot dissociation in colony morphology of som strains of oral lactobacilli. 2. This dissociation is readily reversible, is apparently not selection, and is unaccompanied by gros changes in physiology. 3. Chemical analysi of salivas for tryptophane and indol indicate the association of greater amounts of suc compounds with the caries-immune group 4. Tryptophane-induced rough to smoot variation in lactobacilli is different from th similar dissociation which occurs in saliva o caries-immune individuals.

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Zone Electrophoresis of Intestinal Alkaline Phosphatase.* (22085)

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It has been shown(1) that soluble phosphatase prepared by treating the microsomal fraction of bovine intestinal mucosa with butanol traveled at a discrete peak during zone electrophoresis on filter paper, and that its solubility behavior makes it a promising starting material for further purification. Recently, Roche and Bouchilloux(2) reported purification of dog intestinal phosphatase by electrophoresis on filter paper. Morton(3) reported in detail upon application of acetone fractionation to material obtained by butanol treatment of microsomes from calf intestine, which yielded a highly purified preparation. The present paper deals with the use of electrophoresis in the starch block for purification of bovine intestinal alkaline phosphatase, and with changes in electrophoretic mobility during the purification process. Purity as great as achieved by Morton does not appear to have been reached in our study, but additional information regarding change in behavior of phosphatase during purification has been obtained.

Materials and methods. Crude intestinal phosphatase. Even with a homogenizer designed for treatment of relatively large volumes of material(4), isolation of the microsomal fraction from quantities of starting material required, was cumbersome. Consequently, butanol treatment was applied directly to the intestinal tissue. Thirty-five pounds of calf duodenum, obtained from 40 to 60 animals, were rinsed with tap water, slit, and placed in washing machine with aluminum agitator and porcelain-lined tank. Other exposed metal parts had been replaced with stainless steel and rubber with polyethylene. Five gallons distilled water and 3 gallons n-butanol were added and agitator started. After extracting 30 minutes at approximately 25°C, the intestinal tissue was removed and butanol-water emulsion transferred to glass carboys for storage at –5°C. After 12 to 18 hours, the emulsion had separated into an upper butanol layer, a middle gel layer containing much denatured protein, and aqueous layer containing the enzyme. The aqueous layer was siphoned, and pH adjusted to 4.9-5.0 with acetic acid. Precipitation and equilibration took place for 24 hours at 0°C. The supernatant solution was then siphoned and clarified by passage through a Sharples Supercentrifuge. The inactive precipitate was discarded, and an equal volume of acetone, pre-cooled to –5°C, was slowly added, with stirring, to the clarified solution. The enzyme-containing precipitate, formed upon addition of acetone, was equilibrated at –5°C for 24 hours and then removed by decantation and centrifugation at –5°C. The precipitate of crude enzyme was washed in the cold, twice with 50% acetone and twice with 100% acetone. It was then dried in a vacuum desiccator over sulfuric acid. Average yield of crude, dry enzyme was 23 g. *Starch block electrophoresis.* 1.5 g of crude enzyme were suspended in 7 ml of veronal buffer at pH of 8.6, ionic strength 0.02, and extracted with stirring for 90 minutes at 5°C. The suspension was then centrifuged in Spinco Model L preparative ultracentrifuge 45 minutes at 140000 x g, and supernatant solution recovered. This solution was subjected to electrophoresis in starch according to the method of Kunkel and Slater(5). The buffer for electrophoresis was the same as used in extraction. The starch block was 30 cm long, 5 cm wide, and one cm deep. The voltage gradient was 6.7 v/cm, and the current 7-9 ma. Migration occurred for 12 hours at 0°C. At termination of migration, the starch block was cut into 1 cm segments, each eluted by

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suspending it in 5 ml of pH 8.6 veronal buffer. Supernatant solutions obtained after centrifuging starch suspensions were used for enzyme and protein analyses. *Determination of phosphatase.* The substrate was disodium p-nitrophenyl phosphate, made as a 1% solution in 0.05 M bicarbonate buffer containing 5 x 10^{-4} M magnesium acetate, pH 10.1. Enzymatic activity was measured by incubating 2 ml of substrate solution with 0.05 ml of enzyme solution for 20 minutes at 37°C. The reaction was stopped by addition of 10 ml of 0.002 N NaOH to the reaction mixture. Absorbance of the solution was then measured at 420 mμ, setting absorbance to zero with appropriate reagent blank. Concentration of enzyme was adjusted to produce an increase in absorbance of 0.1 to 0.3. The disodium p-nitrophenyl phosphate was prepared by the method of Bessey and Love(6), and recrystallized by the method of Aschaffenburg(7). A unit of phosphatase activity is that amount of enzyme which will release one millimole of p-nitrophenol in one hour, the specific activity as the number of units per mg nitrogen. Nitrogen was determined by either micro-Kjeldahl digestion and titration, or by the ultra-micro method of Tompkins and Kirk (8). Protein in the eluates from starch blocks was measured by the method of Lowry *et al.* (9). Since color development was carried out directly on eluates, without precipitating the protein, other chromogenic material may have influenced the apparent protein distributions.

Results. The crude material used had a specific activity of 1.5, approximately 30 times greater than the average mucosal homogenate. The dried, crude material was extracted with pH 8.6 buffer, and the extract subjected to electrophoresis in starch with results shown in Fig. 1A. Positions of the peaks are not corrected for endosmotic flow. However, position of the major enzyme peak is essentially that of an immobile component. The minor peak accounts for approximately 10% of the entire enzyme found on the block. After removal from the block, the major enzyme component had a specific activity of 1.9, or 35 times that of the mucosal homogenate, and a 30-minute pre-incubation at 37°C with 0.01 M Mg^{++} and 0.01 M alanine resulted in 1.4 fold increase in activity over that found with out pre-incubation. All specific activities are obtained after pre-incubation with Mg^{++} and alanine. The solution obtained from mid-portion of the major enzyme peak, which contained two-thirds of the total enzyme of that peak, was subjected to a second starch block electrophoresis under conditions identical with those for the first block. These results are presented in Fig. 1B. Although only the middle of the major peak found on Block 1 was used, 2 enzyme peaks were again obtained and the minor enzyme peak of Block 2 represented approximately 25% of the total. Specific activity of the major enzyme component was 4.4, indicating an 83-fold purification when compared with average mucosal homogenate. At this stage, pre-incubation with magnesium and alanine produced a 1.65-fold increase in activity. Distribution of protein on Block 2 was also unexpected, since material was found which migrated more rapidly toward the

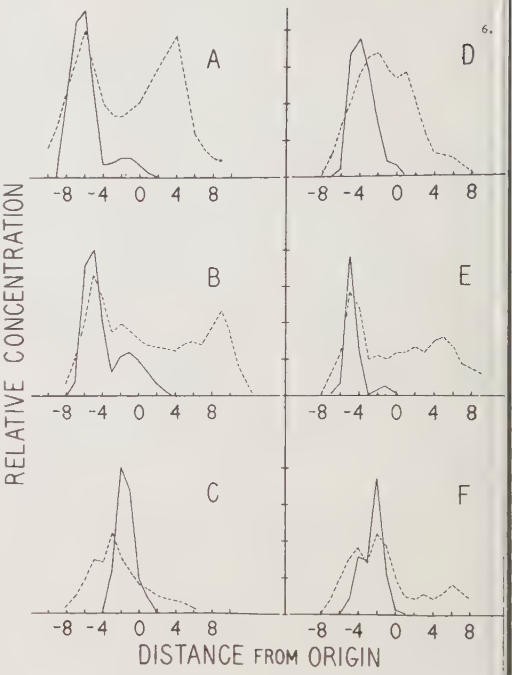

FIG. 1. Distribution of enzyme (solid line) an protein (dotted line) from starch blocks after elec trophoresis for 12 hr at a voltage gradient of 6. v/cm and 0°C. Veronal buffer, pH 8.6, $\Gamma/2 = 0.0$ Distances from the origin (0) are indicated in c toward the anode or –cm toward the cathode. Th concentration scales are arbitrary, and not com parable for the various blocks. See text for details

anode than did the material on Block 1 from which it was taken. The eluate from the section of Block 2, containing the middle portion of the larger enzyme peak, was subjected to electrophoresis a third time, again using same conditions as in the first 2 blocks. These results are shown in Fig. 1C. A single enzyme peak was now found, having the mobility of what had previously been the minor component. The specific activity was 13.7, representing a 260-fold purification. Pre-incubation with magnesium and alanine produced a 6.4-fold increased activity. Protein distribution of the third starch block also differed from that observed on the 2 previous blocks. Although 2 components are discernible, the enzymatic activity does not seem to be definitely associated with either of them. It appears that enzymatically active protein on this block could not have represented more than 30% of the total. During each period of electrophoresis, 40 to 50% of the enzyme placed on the block was lost, as judged by summing enzymatic activities found in individual segments. This was a considerably greater loss than that observed in recovering enzyme added to starch. Combination of eluates from individual sections, in an attempt to reconstitute the original enzyme solution, did not produce any increase in activity over that calculated from the sum of activities in individual eluates. Since storage in solution at 0°C for the same time as required for electrophoresis produced a much smaller loss in activity, it did not appear that losses on storage and in recovery from starch, could amount to more than 15 or 20% of enzymatic activity. The larger loss during electrophoresis remains unexplained. Since the shift in mobility, accompanied by increased response to magnesium activation, suggested that tightly bound magnesium might be lost and contribute to the change in mobility, a second series of starch blocks was studied with 0.001 M magnesium acetate in the buffer. This was again 0.02 M veronal buffer at pH 8.6. The results are shown in Fig. 1D, 1E, and 1F. As before, Fig. 1D is the crude enzyme, now subjected to electrophoresis in the presence of Mg^{++}. The distribution shown in Fig. 1E is that obtained on taking the middle portion of the major enzyme component from the first block and subjecting it to electrophoresis in the presence of Mg^{++}, and the process repeated for Fig. 1F. Although presence of Mg^{++} appears to have suppressed appearance of the more mobile enzyme peak, the same general trend of results is observed as was found for electrophoresis in the absence of Mg^{++}.

Discussion. The results obtained confirm the previous conclusion 1) that alkaline phosphatase in the material obtained from n-butanol treatment of microsomes represents only a small part of the total protein. A similar conclusion can, of course, be drawn from the work of Morton(3) and the many papers of Roche and coworkers. It also serves to emphasize the problems associated with losses in activity during any purification procedures which have been investigated. Alteration in mobility during the series of electrophoretic separations indicates that purification procedures may not simply remove inactive protein, but may result in changes in the enzyme. We do not know whether a similar change would be observed during repeated fractionation with acetone. There seem to be two possible explanations for change in mobility of the enzyme. One possibility would be that the enzyme tends to form complexes with other proteins or ions which are sufficiently stable to yield discrete zones in starch block electrophoresis, but which may become less stable, as components of the original mixture are removed in electrophoretic separation. Magnesium alone is not responsible for this behavior, but magnesium does appear to have some influence on the process as judged by reduced tendency of enzyme to shift into the more mobile component in the presence of magnesium. A serious objection to this is found, however, in the behavior of the non-enzymatic protein. In general, if a complex were being disrupted and giving rise to a more highly negatively charged fraction, a somewhat comparable amount of a more highly positively charged material should be formed. This does not appear to be the case, unless such material has migrated off the block. A second possibility would be that the enzyme is altered by a process of proteolysis, and that some such process is in general responsible for the fact that a portion of one block behaves so differently from what would be predicted when subjected to electrophoresis a second time. Changes in the electrophoretic behavior of enzyme preparations on standing and the degree of reproducibility in the same and different preparations do not, however, support this explanation. The problem requires further study, but we are inclined more to the view that some explanation involving dissociation of a complex provides the answer.

Summary. A crude preparation of alkaline phosphatase from bovine intestinal mucosa, obtained by treatment with butanol to release enzyme from the microsomes, was subjected to sequential electrophoresis on starch blocks. The results indicate that the phosphatase may have been present initially in a complexed form and that the complex was disrupted during repeated electrophoretic separation. Such behavior, together with the rather small amounts of enzyme protein which appear to be present in the starting material, may account for some of the difficulties encountered in attempts to purify intestinal alkaline phosphatase. An approximately 260-fold purification was achieved, but the amounts of final product obtained were too small for further study.

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Effect of Season on Appetite and Food Consumption of the Lizard, *Anolis carolinensis.* (22086)

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The problem of the regulation of food intake by vertebrate animals has received much attention in recent years(1). Among the factors which seem to be implicated in appetite control are the fat stores of the animal and differences between arterial and venous blood glucose levels. Since the lizard, *Anolis carolinensis,* undergoes marked seasonal changes in total body fat as well as changes in both the concentration and distribution of other metabolites(2,3), a study of the food consumption of lizards at different times of the year was undertaken to determine if such changes in composition are correlated with variations in appetite.

Methods. Adult male specimens of *Anolis carolinensis* which had reached the plateau stage of growth were used. Colonies of 8 to 15 specimens, used to determine seasonal changes in food consumption, were formed in different months of the year from lizards recently captured in the New Orleans area. Colonies were kept for duration of each experiment (1.5 to 2.5 months) in small cages in a constant temperature (28° ± 2°C), exposed to natural hours of light (30° latitude) through a north window. An excess of meal worms, the larval form of the insect, *Tenebrio mollitor,* was kept in cages except during the 4-day fasting period preceding body weight measurements. Food intake was determined from the weight of meal worms eaten. Fortunately, large specimens of meal worms are relatively constant in gross composition if they are well nourished and hydrated(4). Samples of meal worms were analyzed periodically and found to consist of 13% ether extractable lipid, 20% protein, 2% ash and 60% water. Using these results and the value of 2% carbohydrate obtained by Mellanby (4), the caloric equivalent of the meal worm diet was approximated as 2 kcal/g of meal worms. This value, although subject to obvious limitations, was used throughout this paper to express results obtained and to equate caloric intake with standard metabolism and fat stores of the animals. To obtain the best possible comparison of appetite in different seasons, the total food consumption during the first month in captivity was averaged and caloric intake was calculated on the basis of original body weight of the animals. Lipid content of droppings was never higher than 2% of the total caloric intake of the colony. At the end of each experiment, animals were sacrificed for organ size and composition measurements. Methods of chemical analysis were the same as those described elsewhere(3).

Results. The standard metabolic requirement of *Anolis,* as calculated from O_2-uptake studies(2), averages 24 cal/g body wt/day. Seasonal variations from the mean are of the order of 10-15%. Energy consumed in excess or deficit of the average standard metabolic requirement is shown in Fig. 1a. Appetite is much greater in summer than in other seasons. For example, animals brought in from the field in June or in July are ravenous in their hunger for meal worms. One large male caught in July consumed 1.2 g of worms within an hour after being placed in the cage. To consume a comparable amount of food, an adult man would have to eat about 40000 kcal at one meal. In contrast to the intense appetite in summer, lizards captured between October and April have little appetite for meal worms. Food eaten by these animals, even at summer temperature of laboratory, is about equal to or less than is required to meet standard metabolic requirements. It is of interest that the nitrogen contained in the food consumed by those colonies of lizards having the poorest appetite was only slightly less than the total N losses of completely starved lizards during the same period(5). In winter lizards reach their peak food consumption 2 or 3 weeks after being brought into the laboratory, whereas animals captured during the remainder of the year exhibit their greatest appetite during the first few days of exposure to laboratory conditions.

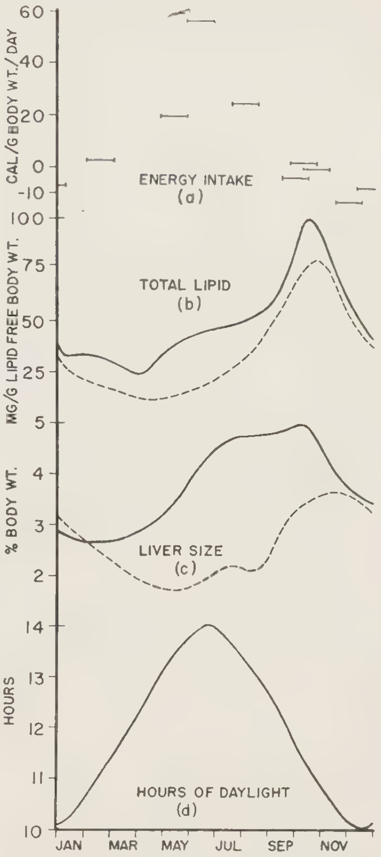

FIG. 1. (a) Seasonal fluctuations in avg energy intake of colonies of adult, male lizards kept in the laboratory at 28°C, exposed to natural hours of light. Each bar shows excess or deficit in calories eaten during the first 30 days of feeding. (b and c) compare total body lipid and liver size of animals at termination of each feeding experiment with values found for animals in the wild. Feeding ————; wild – – – – –. (d) Hours of light between sunrise and sunset at 30°N. latitude.

In Fig. 1b total lipid content of captive animals is compared with that of lizards in the wild. During months of intense appetite, lizards increase in body weight and in total lipid content. The fat storage process occurs throughout the summer but appears to be most efficient during late summer and fall. The time lag between peak appetite and maximum fat deposition may be due to differences in the activity of *Anolis* in these two seasons(6). Calories consumed in excess of standard metabolic requirements exceeded greatly the caloric equivalent of fat stored by mid-May to mid-July captive colony but were only slightly greater than the caloric equivalent of fat stored by mid-July to October colony. From the time of maximum fat storage in the fall until mid-January, appetite is minimal and animals undergo a progressive decrease in body weight and in fat content. Although fat stores have diminished considerably by late December, appetite remains poor. Apparently, in this season, food consumption by the lizard is not directly correlated with lipid stores.

In Fig. 1c liver size of captive lizards after their average 2-month feeding period is compared to liver size of lizards in the wild(2). In late spring, with the return of appetite, the liver enlarges. This enlargement is relatively rapid in animals in captivity where the food is readily obtained and temperatures are always warm. The increase in size of the organ involves a growth as well as an increase in fat. Liver nitrogens of lizards from feeding colonies sacrificed between January and April averaged 0.717 ± 0.151* mg liver N/g lipid free body wt, but by June the average was 0.976 ± 0.159* mg liver N/g lipid free body wt. Apparently, once appetite returns, one of the first important changes in gross composition of the animal is synthesis of liver protein. Once liver protein reaches this peak value it does not vary greatly until the period of hibernation in early winter. The size of livers of captive lizards throughout the year is generally greater than size of organ of animals in the field. As the maximum liver protein in captive lizards in autumn is only slightly greater than the maximum value found in wild controls, the larger liver of captive lizards in this season is due primarily to an increased fat content of the organ.

Discussion. Besides *Anolis,* the lizard, *Eumeces fasciatus*(7), and the alligator(8) are known to have a diminished appetite during the winter. Since *Anolis* does not eat well when brought from the field into a warm laboratory in winter, factors other than mere temperature differences are involved in appetite control in this animal. The possibility that daylength may be such a factor is suggested by a comparison of the chart of energy intake during the different seasons with Fig. 1d which shows the hours of light between sunrise and sunset at different times of the year(9). Preliminary measurements of food consumption of experimental groups exposed to short and long periods of light substantiate this impression(10). It is highly possible that light as well as season of year are further factors which must be considered in any appraisal of the mechanism of appetite control in certain animal forms.

Summary. Food consumption of adult male lizards (*Anolis carolinensis*) at 28°C is maximal in summer and minimal in winter. This seasonal variation in appetite appears to be correlated with hours of daylight and is responsible in part for seasonal changes in body composition of this lizard.

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* Standard deviation from the mean.

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Blood Serum Xanthine Oxidase of Rats Poisoned with Carbon Tetrachloride. (22087)

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In previous studies(1,2) it has been shown that carbon tetrachloride when injected subcutaneously increases the activity of rat liver xanthine oxidase. A strong activation was also observed when this drug was added *in vitro* to the liver homogenate. We were therefore interested to know if the same activation could be detected in the blood serum. Few reports have been published on presence of xanthine oxidase in blood. Older literature dealing with distribution of this enzyme in tissues, is deficient in quantitative data on blood xanthine oxidase(3,4). Blauch *et al.* studying uric acid of rat blood observed that when rat blood is incubated with xanthine an increase of uric acid is obtained due to presence of xanthine oxidase(5). Using a spectrophotometric technic Westerfeld *et al.* determined xanthine oxidase activity of rat blood serum in experimental cancer of the liver and reported values of 50-60 μg of xanthine oxidized/1 ml/hour(6).

Due to the lack of quantitative values for xanthine oxidase and xanthine dehydrogenase of blood serum measured by manometric and colorimetric methods we undertook a series of determinations in normal rats which served for comparison with the injected ones.

Methods. Three hundred male Wistar white rats weighing between 100 to 150 g and maintained on well balanced diet were used. Blood samples were withdrawn by

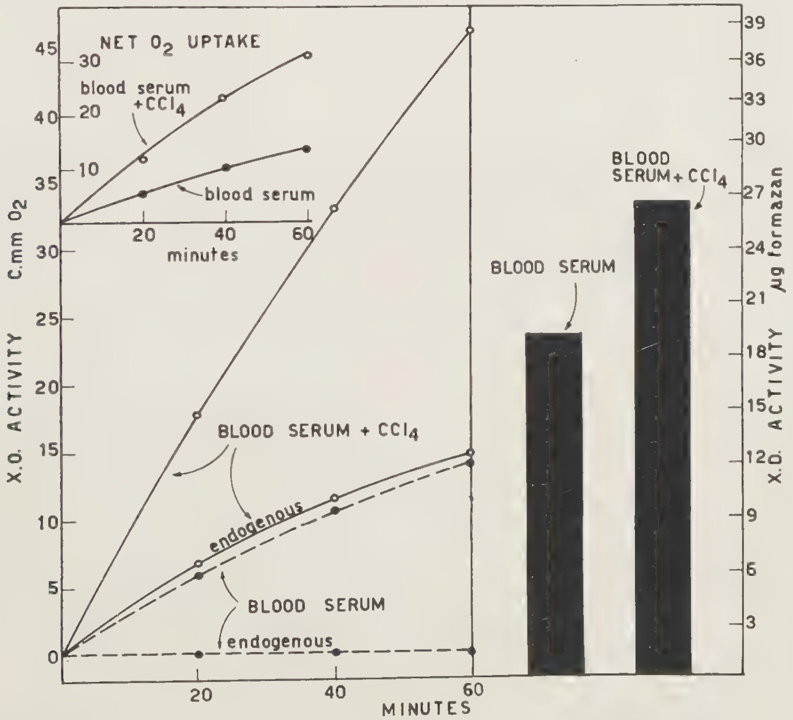

FIG. 1. *In vitro* effect of CCl_4 on xanthine oxidase and xanthine dehydrogenase activity of rat blood serum. Left: oxygen uptake of blood serum in mm^3 measured in Warburg manometers before and after addition of CCl_4. Net oxygen consumption due to xanthine is shown in upper left-hand corner. Right: xanthine dehydrogenase expressed in μg formazan produced/1 ml serum/30 min. before and after activation by CCl_4.

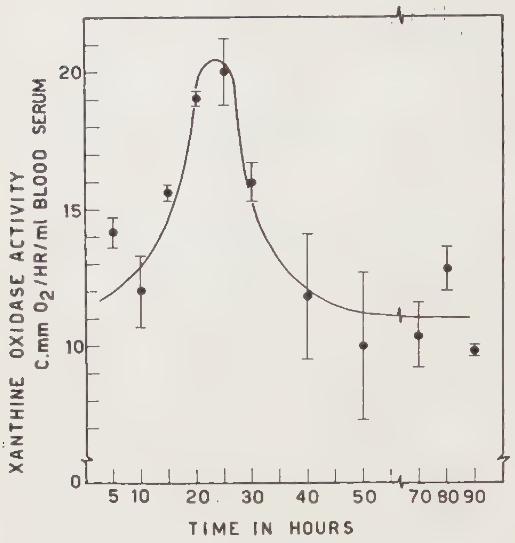

FIG. 2. Blood serum xanthine oxidase activity of rats poisoned with CCl_4. Each point in the graph represents determinations on pooled nonhemolyzed serum from 15 rats (3 groups of 5 rats). Vertical bars indicate avg values ± stand. dev.

heart puncture and each determination was carried out on pooled nonhemolyzed serum from 5 rats. The animals were injected subcutaneously with 0.1 ml CCl_4/100 g body weight and the blood taken at different intervals (5, 10, 15, 20, 25, 30, 40, 50, 70, 80 and 90 hours) after injection. The coagulated blood was centrifuged, the serum separated and used immediately for analysis. Hemolyzed samples were discarded since it was observed that hemolysis interferes with reduction of the triphenyltetrazolium dye. Xanthine oxidase activity was measured manometrically as previously described(1) using 1 ml of blood serum, 0.2 ml of 0.05 M xanthine solution in 0.05 M NaOH and enough pyrophosphate buffer pH 8.6 to give a final volume of 2.0 ml. Xanthine dehydrogenase activity was measured colorimetrically by the method of Villela(7) using 1 ml of blood serum, 0.1 ml of 0.05 M xanthine solution, 0.3 ml of 0.1% triphenyltetrazolium chloride and 2.0 ml of pyrophosphate buffer pH 8.6. *In vitro* experiments were carried out by adding 0.1 ml CCl_4 to 10 ml blood serum, mixing well for 10 minutes and then centrifuging. Determinations were made in the CCl_4 free supernatant layer.

Results. Normal values were obtained from the blood serum of 55 rats. The average values together with the range plus or minus the standard deviation showed values of 12.9 ± 0.86 cmm O_2/ml serum/60 minutes for xanthine oxidase activity and 17.81 ± 3.00 μg formazan/ml serum/30 minutes at 37°C for xanthine dehydrogenase activity. Each determination was carried out on pooled serum from 5 rats.

In vitro activation of rat blood serum xanthine oxidase and xanthine dehydrogenase by CCl_4 is shown in Fig. 1. Values of 120% activation for xanthine oxidase and 38.5% for xanthine dehydrogenase activity were observed.

The enzyme activities increased in animals injected with CCl_4, the highest values were found 20-25 hours after injection of the drug (Fig. 2). Thereafter, the enzyme activity for xanthine oxidase and xanthine dehydrogenase returned to the normal level.

Discussion. Bruns and Neuhans showed that aldolase and phosphohexose isomerase increased in serum of mice poisoned by intraperitoneal administration of carbon tetrachloride(8). The highest activity was reached 18 to 20 hours after injection of the drug. However, the same enzymes decreased in liver, reaching lowest values 50 hours after injection. These authors suggested that a loss of these enzymes from liver tissues could be the cause of the sharp increase presented in the serum.

It was demonstrated that CCl_4 activates strongly the xanthine oxidase of the rat liver a few hours after subcutaneous injection of the drug(1). Xanthine oxidase was also activated in the blood serum 20 hours after the single subcutaneous injection of carbon tetrachloride as shown in Fig. 2. The simultaneous increased activity of this enzyme in the liver and blood serum indicate that a different mechanism than that proposed for aldolase and phosphohexose isomerase must be responsible.

The distribution of xanthine dehydrogenase was recently studied in the serum of normal rats. It was found that enzyme activity is confined to the lipoprotein fractions(9), suggesting that the activation by CCl_4 could be attributed to the split of the linkages between the enzyme and the lipids as observed when milk(10) and liver xanthine oxidase(2) are treated by other organic solvents.

Summary. Quantitative determinations of xanthine oxidase and dehydrogenase activity in blood serum of normal rats were made. It was shown that a single injection of 0.1 ml CCl_4/100 g body weight is able to activate the blood xanthine oxidase and dehydrogenase, reaching the highest values 20-25 hours after injection. However, after 35 hours the values returned to normal. Activation of the enzyme was also obtained *in vitro* by adding CCl_4 to a sample of serum of known enzyme content.

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Recently Classified Types of Coxsackie Virus, Group A. Behavior in Tissue Culture. (22088)

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In addition to the 10 previously described types of Coxsackie virus, Group A(1,2), 9 new types have now been assigned numbers. The representative strain of Type 11 is the Belgium 1 strain of Godenne and Curnen (3), that of Type 12, the Texas 12 of Contreras, Barnett, and Melnick(4). Type 13 was isolated in this laboratory from fecal specimens from Mexico. Type 19 was received from Dr. R. J. Heubner, and the remainder from Dr. J. H. S. Gear of the South African Institute for Medical Research(5). All are viruses which induce flaccid paralysis in suckling mice but no apparent disease in older mice and are characterized by the production of marked muscle degeneration but no significant lesions in the central nervous system in the experimental animal. With the exception of Types 12 and 14 all resemble Types 1, 7, and 9 in low infectivity of brain suspensions for suckling mice. Passage and testing with muscle suspension are necessary. In neutralization tests in suckling mice there is definite, although moderate, reciprocal cross reactivity of A-11 with A-15 and of A-13 with A-18. As noted by Contreras(4), A-12 cross reacts with A-5. Regular cross reactions may also be found with A-8 and A-12, more observable when brain tissue is used in neutralization tests. Slight cross reactivity of A-14 in A-7 serum, A-15 in A-17 serum, and A-17 in A-11 serum has been seen.

Tissue culture. Methods. Human uterine tissue and trypsinized monkey kidney tissue cultures were prepared as previously described (6). Trypsinized monkey testicular cultures were made similarly to the kidney cells. HeLa cell cultures were supplied by the Laboratories for Virology under the direction of Dr. Irving Gordon. On receipt, the nutrient fluid containing human serum was removed and replaced by 1.5 ml of the maintenance fluid of Scherer, Syverton, and Gey(7) without added serum. The tubes were rotated so that the entire inside surface was washed and were then allowed to stand upright for a minimum of one-half hour. The washings were removed and replaced by 1.0 ml of mainten-

TABLE I. Cytopathogenicity of Coxsackie Virus.

Coxsackie virus		Monkey cells trypsinized		Human cells	
Group	Type	Kidney	Testes	Uterine plasma clot	HeLa cells
A	9	+	+	+	—
	11	—	—	+	+
	13	—	—	+	+
	15	—	—	+	+
	18	—	—	+	+
B	1	+	+	—	+
	2	+	+	—	*
	3	+	+	+	+
	4	+	+	—	(+)
	5	+	+	+	+

* Only one strain regularly cytopathogenic under the given conditions.

(+) More erratic than B-1, 3, and 5.

ance fluid. All types not cytopathogenic for these cells were retested on HeLa cells that had been given two further washings of 1.0 ml each. No additional cytopathogenic strains were encountered. In early tests 10% chicken serum was used in the maintenance fluid for the test and later, 2% calf serum. For many of the results recorded no serum was added. Three tubes each were inoculated with 0.1 ml of the test material. For the cultures grown on glass the inoculum was added to the tube after the maintenance fluid; for plasma clot cultures the tissue was inoculated and let stand at room temperature for one-half hour before the addition of the maintenance fluid. Neutralization tests were made, in general, as previously described(6).

Results. Trypsinized monkey kidney cell cultures. All types of Group B were cytopathogenic for monkey kidney cells, but only Type 9 of Group A(8,9). None of the newer Group-A types induced evident cell damage (Table I).

Trypsinized monkey testicular tissue cultures. In previous tests on stationary plasma clot cultures of monkey testicular tissue with 3 strains of A-7, 2 of A-9, one each of B-1, B-4, and B-5, and 6 of B-2, cytopathogenic activity was obtained with the A-9 strains only. Steigman obtained cell damage with 2 strains of B-2 on roller tubes of plasma clot cultures with fluid 199 as maintenance medium(10). Using the trypsinized cells, we have found that the same types cytopathogenic for monkey kidney cells induce cell damage in the testicular tissue grown on glass. With the Group-B types the effect was more prompt and more marked. The newer Group-A types were not cytopathogenic.

Human uterine tissue (plasma clot). Among the previously numbered types Group A, Type 9, some strains of Group B, Type 3, and Type 5(6) were found to be cytopathogenic for human uterine tissue. Four of the new types in Group A, Types 11, 13, 15, and 18, are also cytopathogenic. Cell damage may appear in from 3 to 7 days but is usually definite at 5 days. All are neutralized by their specific immune sera. Two of 4 strains of A-14 caused minor cell damage in early passages on this tissue and also on plasma clot cultures of monkey testicular tissue. This, however, could not be established on passage.

HeLa cells. Our experience with the previously numbered types has been, in general, similar to that of Crowell and Syverton(11). Group A, Types 1-10, were not cytopathogenic. Strains of Group B, Types 1 and 3, are cytopathogenic, as is Type 5. More difficulty was experienced with B-4. In earlier trials reactivity was erratic but more recently we have been able to make type determinations of a number of strains on HeLa cells, although

TABLE II. Neutralization Test in HeLa Cell Tissue Culture Coxsackie Virus, Group A, Type 15.

Test dose				
	Serum		A-15 No. 52108	
Virus	Kind	Dilution	5 days	6 days
10^{-2}	Maintenance medium		2+ 2+ 2+	4+ 4+ 4+
10^{-3}			2+ 2+ 2+	4+ 4+ 4+
10^{-4}			— ? —	2+ 4+ 2+
10^{-5}			— — —	— — —
10^{-1}	Normal hamster	5	2+ 2+ 2+	3+ 4+ 4+
	A-11	5	— ? —	1+ 3+ —
		10	— — 1+	2+ 2+ 3+
		20	2+ 2+ 2+	4+ 4+ 4+
	A-15	10	— — —	— — —
		40	— — —	— — —
		160	— — —	— — —
		640	2+ 1+ ?	4+ 3+ 3+
Uninoculated controls			— — —	— — —

— = same as control uninoculated tissue; ? = slight damage, specificity doubtful; 1+ = small but definite area of damage; 2+ = large areas of definite damage but with large remaining areas of normal cells; 3+ = extensive destruction; 4+ = complete destruction.

endpoint titers are not high. With B-2 only one strain of 10 tested was consistently cytopathogenic on HeLa cells. Two other strains continue to give erratic results on repeated transfer. Among the new Group-A types the 4 which are cytopathogenic for human uterine cells, Types 11, 13, 15, and 18, are also cytopathogenic for HeLa cells. Beginning cell damage may be seen in from 2 to 6 days. Satisfactory neutralization tests may be made with these strains. The cross reactivity noted in the mouse neutralization tests is also evident in tissue culture (Table II).

Summary. Nine additional types of Coxsackie virus, Group A, have been assigned numbers, Types 11-19. Strains of 4 of these types, Types 11, 13, 15, and 18 are cytopathogenic for human uterine tissue in plasma clot culture and for HeLa cells, but not for trypsinized monkey kidney or testicular cells.

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Association of I^{131}-Labeled Lactogenic Hormone with Cytoplasmic Nucleoprotein of Mammary Gland Cell.* (22089)

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It has been reported from this laboratory (1) that lactogenic hormone labeled with I^{131} becomes associated predominately with the cellular particulates following intraductal injection into the rabbit mammary gland. It was suggested that this study indicates the importance of the mitochondria and microsomes in the synthetic activity associated with milk secretion and that the site of action of the lactogenic hormone at the cellular level is in association with the mitochondria and microsomes of the secretory cells. Studies of the nature of this association of the lactogenic hormone with the mitochondria and microsomes would be expected to yield information basic to an understanding of the mode of action of the lactogenic hormone at the cellular level. To this end, studies of the chemical nature of this association have been carried out.

Due to the nucleoprotein and lipoprotein nature of the cellular particulates this investigation has been limited to study of the involvement of two chemical moieties, the pentosenucleic acid and the lipid, in the association of the lactogenic hormone with the mitochondria and microsomes. Demonstration of the involvement of particulate lipid or nucleic acid in the lactogenic hormone-particulate association would at least indicate something of the nature of this association and to that

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extent add to the understanding of the basic biochemical mechanisms involved.

Methods and materials. The method which was used for the labeling of the lactogenic hormone with radioactive iodine has been described(1). Experimental treatment of the animals and preparation of the samples for counting of sample radioactivity were also described(1). For all studies reported here an isotonic sucrose homogenate of the mammary gland from pseudopregnant rabbits, which had been injected intraductally with radioactive iodine labeled lactogenic hormone was centrifuged at 1000 g for 10 minutes to remove nuclei and the supernatant from this centrifugation was used for fractionation. Grinnan and Mosher(2) used 4 M guanidine hydrochloride for the production of a highly polymerized and apparently undenatured RNA from liver. This same procedure has been used in this study for the precipitation of nucleoprotein from the mammary homogenate. A second procedure for precipitation of nucleic acids and nucleoproteins has been studied by Berkman, Housewright and Henry (3) and Euler and Heller(4). These studies indicated that streptomycin was effective in precipitation of cytoplasmic nucleoproteins. In the present work the homogenate, as prepared above, was brought to pH 7.0-7.4 and streptomycin added to a final concentration of M/200 to M/400, held at room temperature for one hour with occasional shaking, chilled to 0°C for one hour and the nucleoprotein precipitate removed. Two methods which have been used for the solubilization of particulate proteins were studied for their action on the lactogenic hormone-particulate association. Wainio *et al.*(5) studied the solubilizing effect of two to four percent desoxycholate on the cytochrome oxidase associated with the insoluble particulates of heart muscle preparation. Morton(6) studied the effect of excess n-butyl alcohol on the solubilization of enzymes associated with insoluble cytoplasmic particulates. The isotonic sucrose homogenate with the nuclei removed was treated for three hours at room temperature with these reagents, centrifuged at 25000 g for 90 minutes, dialyzed against distilled water and the streptomycin precipitable material counted as being the material taken into solution by these procedures. The results obtained with these methods are of interest due to the apparent dependence of these methods on the removal of lipids and the rupture of lipoprotein bonds.

TABLE I. Precipitation of Activity from Unfractionated Particulate Preparations.

4M guanidine HCl precipitation			Streptomycin precipitation		
Ppt., cpm	S'nat., cpm	% ppt.	Ppt., cpm	S'nat., cpm	% ppt.
243867	120070	67.0	476015	113406	80.7
71531	53388	57.2	206666	35995	85.1
76482	29284	72.3	111549	14886	88.2
206240	36873	84.8	82860	10702	88.5
601242	115590	83.8			
375056	137538	73.1			
1850	1556	54.3			
27806	4988	84.7			
152759	11921	92.7			
Avg % ppt.	74.4 ± 14.2			85.6 ± 10.3	

Results. The studies reported here for the distribution of radioactivity following precipitation of the nucleoprotein with guanidine hydrochloride or streptomycin indicate that the cytological localization of the lactogenic hormone noted previously is, in fact, an association of the lactogenic hormone with the nucleoprotein of the cytoplasm.

These data (Table I) indicate that precipitation with 4M guanidine hydrochloride precipitates approximately 75% of the lactogenic I^{131} hormone as nucleoprotein. Approximately 85% of the lactogenic I^{131} is precipitated with streptomycin. It is seen that the agreement between the two methods is rather good, with at least 75% of the radioiodine activity labeling the lactogenic hormone being precipitated as the nucleoprotein by these methods.

The experiments using desoxycholate or n-butyl alcohol in attempts to solubilize the labeled lactogenic hormone-particulate association have given essentially negative results (Table II). These data, while not extensive, indicate by their negative nature that the particulate lipids are probably not involved in the hormone-particulate association.

Discussion. Basic to an understanding of

TABLE II. Solubilization of Particulates as Measured by I^{131} Activity of Non-Dialyzable, Streptomycin Precipitable Material.

	4% desoxycholate	Excess n-butyl alcohol	
	No. 1	No. 1	No. 2
Cpm soluble	17760	9450	7953
Cpm unfractionated particulate preparation	423620	423620	292035
% soluble	4.2	2.2	2.7
% insoluble or dialyzed	95.8	97.8	97.3

the mode of action of a particular hormone will be information which indicates where and how the metabolism of the target cell is controlled by the hormone. These studies appear to implicate the pentosenucleoprotein of the cytoplasmic particulates as being important in the action of the lactogenic hormone on the secretory cells of the mammary gland. Little is known of the function of the nucleic acid and nucleoprotein of the cytoplasm but it has appeared that they are involved in the synthetic processes of the cell, particularly those in which the cytoplasmic particulates are involved. It may be postulated that the association of the lactogenic hormone with the cytoplasmic particulate nucleoprotein results in changing the conditions of enzyme attachment to the particulate nucleoprotein or in changing the activity of the enzymes which are already in association with the nucleoprotein. Such changed conditions of enzyme attachment, affecting the enzymatic composition or activity of the cytoplasmic particulates, could result in changing the direction of the synthetic activity of the cell as occurs with the initiation of lactation.

Summary. Studies have been carried out with lactogenic hormone labeled with radioactive iodine in pseudo-pregnant rabbit mammary gland which indicate that the labeled hormone becomes associated with the cytoplasmic particulate nucleoprotein. Other data suggests that the particulate lipids are probably not involved in the lactogenic hormone-particulate association. These findings have been discussed in terms of their relation to the shift in synthesis which occurs in the mammary gland with the initiation of lactation.

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Carbon and Water Contents of Mouse and Rat Tissues. (22090)

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In reporting tissue analyses for radiocarbon, tracer chemists have employed a wide variety of units (*e.g.* per cent of injected dose, specific activity of tissue carbon, total activity per organ, activity per unit weight of fresh or dried tissue, etc.). Sometimes the choice is clearly dictated by the immediate context of the measurement; at other times the units are those most conveniently derived from the chosen method of radioassay (*e.g.* specific activity when $BaC^{14}O_3$ is assayed at "infinite thickness"). In analyzing and comparing data from several published sources, the reader must frequently translate from one set of units to another. Adequate information, however, is seldom presented for even an approximate conversion.

Table I records soft-tissue concentrations of water and of organic carbon in mice and rats from this laboratory. The data were obtained in the course of a study on the metabolism of C^{14}-labelled atropine(1). All values were presumably influenced by the genetic and nutritional histories of the test animals. Insofar as these mice and rats are representative of those in general laboratory use, the information in Table I makes it possible to transform, among the following reference systems, any C^{14} concentrations which are known in any one of these systems: activity per mol of tissue C, per unit weight of wet (fresh) tissue, or per unit weight of dried tissue. Unlike the water data, the tissue carbon values are thought to be unique in terms of their accuracy and precision (*cf.* 2), illustrating that the carbon method described by one of us(3) is particularly useful for tissue samples.

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TABLE I.* Carbon and Water Contents of Mouse and Rat Tissues.

	Water (% wet wt)		Carbon (% dry wt)	
	Mouse	Rat	Mouse	Rat
Adrenals	—	—	66.2	58.4
Gall bladder & bile	—	—	56.9	—
Pancreas	—	71.6	55.0	53.7
Skin (hair sheared)	—	65.5	54.6	53.7
Spinal cord†	70.7	71.6	59.3	56.9
Medulla & brain stem†	73.3	77.1	55.5	57.1
Cerebellum†	76.8	78.4	55.6	54.1
Cerebrum†	77.2	78.8	54.2	54.5
Liver	70.5	75.6	53.5	52.2
Blood (heart)	—	—	52.6	51.1
Kidneys	74.7	75.7	52.1	51.0
Thymus	76.1	—	53.6	—
Genital apparatus‡	—	75.4	52.2	51.0
Submaxillary gland	—	76.9	52.8	48.6
Parotid gland	—	78.2	—	48.9
Urinary bladder	70.8	74.0	54.2	52.9
Diaphragm	76.4	76.8	52.3	50.4
Heart	75.5	78.2	51.8	49.6
Hamstring muscles	77.0	77.6	51.2	48.4
Stomach§	78.4	78.9	51.8	49.9
Colon & caecum§	79.5	79.4	51.3	49.7
Duodenum & jejunum‖	77.4	77.5	49.5	48.9
Ileum‖	78.2	76.4	48.5	48.5
Lungs	78.3	79.7	51.6	49.4
Spleen	75.8	77.7	49.6	48.6
Testes	82.0	86.2	50.0	48.1
Colonic contents (feces)	—	72.5	37.9	30.5

* Conventional methods (5) were used to calculate stand. deviations for determinations of carbon and water in mice and rats. These 4 stand. deviations were found unexpectedly to be almost equal, namely about 2% units. Because almost every value in the table is based on 12 animals, the estimated stand. error for each of the means is 0.6. The stand. error of the difference between two means in the same column is approximately 0.8.

† The rat data (only) represent here 5 analyses each (stand. error of the difference between means about 1.3).

‡ Genital apparatus included epididymi, vasa deferentia, seminal vesicles, and part of the prostate.

§ Stomach, colon, and caecum were emptied of contents and mucosal surface was gently rinsed with isotonic saline.

‖ Analyses of duodenum, jejunum, and ileum included entrapped secretions and small amounts of chyme.

Materials and methods. The data were derived from 12 male white mice of mixed breed, ranging in body weight from 26.8 to 38.7 g (mean 33.3 g), and from 12 male albino rats with body weights between 106 and 153 g (mean 128 g). The rats were obtained from a colony bred in these laboratories(4) from an original Wistar strain. After weaning, both mice and rats were maintained on a Purina Rat Chow plus cooked fresh meat and mixed grain (corn and wheat, with oats as a binder) 3 times weekly. They were deprived of food for 24 to 48 hours before sacrifice, but water remained available *ad libitum.* Some of the animals received atropine one-half hour to 24 hours before sacrifice, but these injections had no apparent effect on the results reported here. Animals were killed by chloroform inhalation, and selected tissues were removed promptly for weighing and desiccation. As soon as each organ was dissected free, a weight was quickly obtained on a torsion or knife-edge microbalance. Because evaporation was not controlled, weights of the smaller organs (*e.g.* adrenals, gall bladder) were unreliable, and consequently no values of moisture content are presented for these organs. All tissue samples were dried *in vacuo* (pump operating continuously) over Drierite for a period of 24 hours at room temperature. Only negligible losses of water could be detected during additional periods of desiccation. After reweighing, the specimens were ground to fine powders; aliquots weighing 2 to 25 mg were subjected to high-temperature combustion and analysis by the method of Gabourel, Baker, and Koch(3). Standards (*e.g.* CP sucrose) were burned daily to check the carbon analyses.

Results. Tissue analyses are summarized by the means in Table I. Almost every mouse tissue had a slightly lower water content and higher carbon content than the corresponding rat organ. Within each species, tissues rich in carbon (*e.g.* near the top of Table I) were generally poor in water; the converse was equally true.† From this inverse correlation and from the observation that tissues of grossly fat mice were invariably richer in carbon than those of lean mice, we infer that variations in tissue carbon reflect chiefly differences in the tissue lipid content, relative to the stores of protein and carbohydrate. This impression is supported by the invariably high carbon level in brain (lipid-rich) and in bile and adrenal gland (sterol-rich). Of course any material with a large inorganic ash is correspondingly deficient in carbon (*e.g.* feces). Analyses on gross subdivisions of the central nervous system suggest that concentrations of carbon and water vary progressively and reciprocally along the cerebrospinal axis. Probably these variations among portions of the brain and cord parallel the ratio between carbon-rich white matter and water-rich grey matter.

Summary. Soft-tissue concentrations of organic carbon and of water were measured in male rats and mice. Both the method and results (Table I) are thought to be useful to the radiocarbon biochemist.

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† Because carbon is expressed only in terms of the dry-tissue weight, this inverse correlation is not fatuous.

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Analysis of Micro Quantities of Sweat for Sodium Chloride by Conductivity Method.* (22091)

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The collection and analysis of small quantities of sweat is an important tool in assessing the mineraloid activity of the adrenal cortex. However, the small amounts which are collected by the most practical technics(1) make analysis of the diluted material by flame photometry or chemical means quite difficult. Even if meticulous care is taken to thoroughly clean glassware, small amounts of sodium are extracted from the glass itself(1). To prevent this, the glassware must be silicone coated and the water freshly distilled. Special precautions must be taken in the handling of the unknowns. This imposes further limitations on the value of the sweat test for clinical and investigative use. To obviate the difficulties inherent in the chemical analysis of such dilute quantities of sodium, the use of radioactive sodium has been suggested(2). The concentration of chloride in the sweat increases with the rate of sweating and parallels the concentration of sodium. Either chloride or sodium can be used as a measure of adrenal cortical activity in the absence of an abnormal salt intake. The potassium concentration falls as the perspiration rate increases(3). But the relationship of the fall of potassium to the rise in chloride is a linear one. Because of the linear relationship between the excretion of sodium, potassium and chloride, it seemed feasible to

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FIG. 1. Henry cell with small volume capacity. Wire leads are placed into side arms filled with mercury.

measure total electrolytes by the conductivity method and express them as NaCl since this element constitutes well over 90% of the total electrolyte concentration of rapidly secreted sweat.

Method. Conductivity was measured by a resistance measuring device which is modification of the Wheatstone Bridge.† This instrument has a sensitivity capable of determining one part of sodium chloride in two million parts of water. A special Henry type conductivity cell was constructed with a volume of only 5 cc total (Fig. 1).‡ It has a cell constant of approximately 0.1. It is cleaned with detergent and rinsed with distilled water. It is dried by washing with alcohol and ether or acetone. There is no necessity to obtain highly purified distilled water since the initial conductivity of the water is determined and is subtracted from the conductivity of the water after the sweat has been introduced. This difference is due to conductivity of the sodium chloride in the sweat itself. We have found that more stable readings can be obtained when the water in the cell initially contains a very small amount of sodium chloride. Also, the solution in the Henry cell can be used over again after making one determination since the blank reading is subtracted from the final reading. If the same solution is used over again, care must be exercised lest the concentration of electrolyte exceed the range of the cell being used. Sweat is collected in a 10 λ pipette and diluted with 5 cc of distilled water in the Henry cell. The content of the pipette is thoroughly transferred by rinsing with the distilled water in the cell. A Henry cell has the advantage over a dip type of cell in that it allows for complete transfer without spillage. The dilution technic described allows for a final accuracy within 3% if the blank value for the distilled water is subtracted.

Results. Utilizing the same dilution (1/500 as given above) a chart can be constructed which relates the observed conductivity of the diluted material to the actual concentration of sodium in the sweat. Known standards of sodium chloride were prepared and diluted 1/500. The initial conductivity of this water

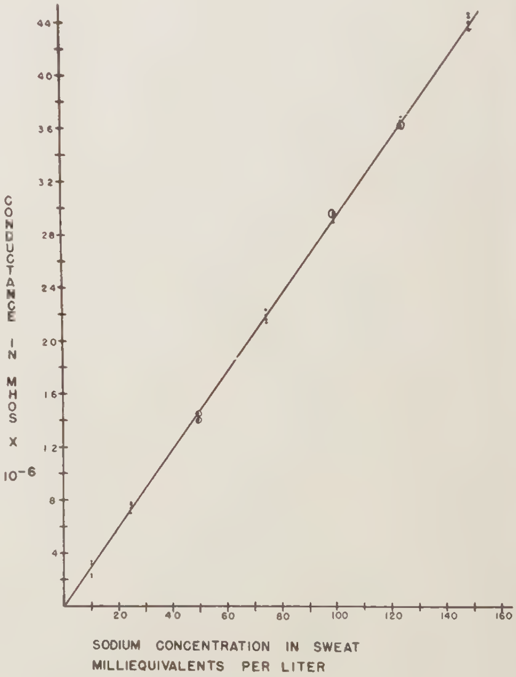

FIG. 2. Relationship between conductivity in Mhos and sodium chloride concentration of 1/500 diluted sweat. Initial (blank) conductivity of water has been subtracted from observed value.

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† Leeds Northrup Model No. 4866.

‡ Made for us by Delmar Scientific Laboratories, Chicago, Ill.

was subtracted from the final reading so that this increased conductivity measured in Mhos was directly related to the sodium chloride concentration. Fig. 2 was prepared from determinations of known concentrations of sodium chloride. There is a linear relationship between conductance§ and sodium chloride concentration.

The standard error was calculated from 20 determinations on a standard solution containing 125 me/L. The dilutions were made with the micropipette in a manner identical to the handling of actual sweat. The deviations of these determinations were used to calculate the standard error of the mean. The standard error of the mean was 3.8×10^{-7} Mhos which corresponds to less than 4 milliequivalents. These determinations were done with a dip cell. The accuracy has been further increased by the use of the Henry cell.

Summary. The fact that the ionic content of rapidly secreted sweat consists almost entirely of sodium chloride make feasible use of conductivity measurement to determine the sodium chloride content of micro quantities of sweat. This method has a high degree of accuracy and is at the same time simple and rapid.

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§ Conductance is the reciprocal of ohms and is expressed in mhos.

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A Method for Collection of Micro Quantities of Rapidly Secreted Local Sweat.* (22092)

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In many diseases it is recognized that disturbances in salt and water metabolism occur(1). It is not known whether the salt retention or losses which are seen as manifestations of some diseases are caused by variations in the minute to minute blood levels of mineraloid hormone activity. These gaps in our information arise from the fact that there is no simplified clinical and investigative tool for testing the overall activity of the salt hormones of the adrenal cortex and other organs. Urinary hormone assays of aldosterone can at best measure only gross changes which occur over a 24-hour period. It is a painstaking laboratory examination which can be done only in a few specially equipped research laboratories. The demonstration by Conn(2) that the concentration of sodium or chloride in perspiration mirrors the mineraloid activity of the adenal cortex provided the basis for the achievement of such a practical test. Most investigators have used hot rooms and other forms of thermal stimuli to produce diaphoresis, and have collected the sweat from an extremity in a water tight envelope made of plastic or thin rubber. The difficulties inherent in this method of sweat collection are obvious. Aside from the difficulties of collection, the electrolyte concentration of sweat has been shown to be dependent upon the rate of sweating. For this reason, it is best to measure the electrolyte concentration at the maximal rate of sweat production(3). It is also essential to be able to take frequent collections of small samples which are unmixed with a large dead space volume of a different concentration. Dole *et al.*(4) have devised a small chamber which contained an absorbent filter paper. Sweat was induced in the skin under the chamber by the injection of a parasympatheticomimetic drug in this area. While this has greatly simplified the collection of

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* Supported by a grant from Schering Corp.

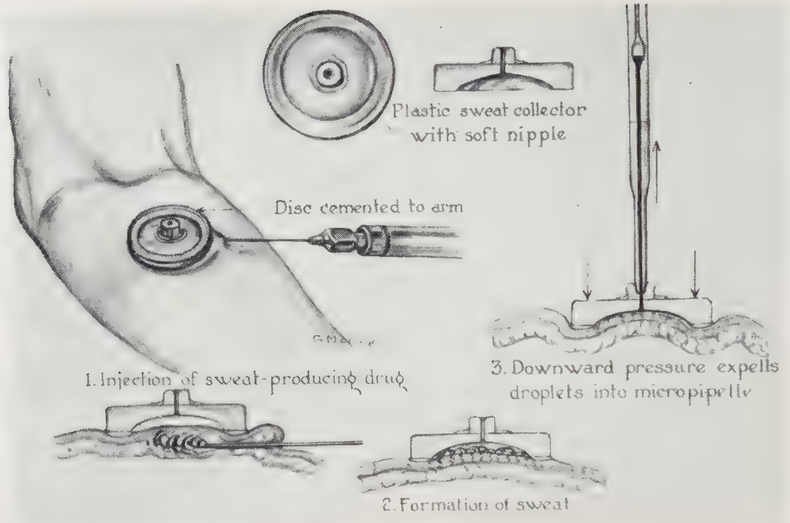
Plastic sweat collector
with soft nipple
Disc cemented to arm
1. Injection of sweat-producing drug
3. Downward pressure expells droplets into micropipette
2. Formation of sweat

FIG. 1. Demonstration of manner of induction and collection of small quantities of sweat. The design is somewhat different than illustrated in the top center cross-section above. The walls of the cavity are convex rather than concave.

small amounts of sweat, only 10 mg of sweat are collected in about 20 minutes. Even with the best precautions in handling, and with precautions in the prevention of evaporation, it is difficult to achieve a high degree of accuracy when handling such minute quantities. The filter paper is then pulped to extract the sodium from it and hence a high degree of dilution of the collected sweat is required.

Materials and methods. Sweat is induced by local injection of cholinergic drugs. Although we originally used only acetyl beta methyl choline the solution suggested by Dole is excellent. Its formula has been slightly modified by the use of saline rather than distilled water in a more convenient amount: Acetyl beta methyl choline (Mecholyl), 25 mg; procaine, 20 mg; sterile isotonic salt solution, 10 ml. Sweat is collected in a small round plastic disc with a central cavity. A capillary hole communicates with the center of the concavity. The diameter of the concavity is one inch and its depth ⅛ inch. This covers a collection area of 5 cm^2 of skin when the collection chamber is cemented onto the skin. The sweat which collects in the concavity can be seen through the transparent plastic, but is protected from evaporation until collection is desired. On the outer flat surface a small circular piece of rubbery vinyl chloride plastic (Tygon or Koroseal) is cemented so that it coincides with the capillary hole in the lens. The completed assembly is shown in Fig. 1. The design now used is slightly different than illustrated. With concave dome-like collection chamber as illustrated, the sweat and bubbles were sometimes trapped at the periphery of the chamber by occlusion of the capillary hole by the skin. If the sweat collecting chamber has converging (convex) side walls, this does not occur. The vinyl chloride plastic teat is made to accommodate the tip of a λ pipette ($\lambda = .001$ ml). The type of pipette best suited for this purpose is one which is calibrated to contain rather than to deliver the required amount. The pipette is then washed out with the diluting fluid to make certain the transfer is complete. For routine work 10 λ pipettes are used although they may be purchased in any size up to 500 λ. Graduated pipettes are ideal for determining secretory rates. Although not recommended for this use, ordinary hemoglobin pipettes are 20 λ (20 cmm or .02 ml) and some have graduations at 10 λ and 20 λ.

Sweat is collected from a smooth and flat flexor surface of an extremity or more usually the back. If there is abundant hair, the site should be first shaved. The skin is then cleansed with a 1/1000 solution of aqueous zepharin and thoroughly rinsed with distilled water. The skin is then dried by blotting and rubbed off with acetone. This process degreases the skin and allows for better adhesion of the plastic disc. The chamber is cemented onto the skin by moistening the outer edge of the disc with "Duco Cement." When the rim is tacky (not fluid), it is pressed against the prepared skin site and held for 3 to 5 minutes.

After fixation of the disc, the skin outside the disc punctured with a fine gauge 1½-inch needle, the point of which is directed toward the center of the disc 0.5 ml of the diaphoric substance is injected subcutaneously (Fig. 1, 1,) with a tuberculin syringe. Local diaphoresis is rapid and copious. In 5 minutes an excess of 20 λ has usually been secreted.

When it is desired to collect the sweat, the arm is placed so the air bubble is near the center of the disc. The tip λ pipette is engaged with the vinyl plastic bottom and gentle downward pressure exerted on the disc. This causes the skin to bulge into the sweat collecting chamber and expresses the sweat into the pipette (Fig. 1, 3). It is best to slightly over fill the pipette and to remove the excess fluid from the tip by capillary action. If this is done, the tip should be wiped with a silk rag rather than filter paper or cotton which may be too highly absorbent and remove the excess too rapidly. Automatically self-filling pipettes are also available from most laboratory supply houses. The sweat is then transferred to the dilution fluid and prepared for flame photometry, or other chemical analysis. We have used the conductivity method to determine sodium. It is described in the preceding paper.

Summary and conclusion. A simplified method of collecting and measuring small quantities of rapidly secreted sweat is described. This method employs local chemical induction of sweating and the collection of this sweat in a hollowed out plastic chamber. The sweat can be transferred to a λ pipette by expressing the sweat through a capillary orifice in the chamber.

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An Antimalarial Effect *in vitro* of Parapyruvic Acid. (22093)

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When preparations of the bird malaria parasite *Plasmodium lophurae* were removed from their host duck erythrocytes and maintained extracellularly *in vitro,* their survival and development were favored by the addition to the culture medium of both adenosinetriphosphate (ATP) and sodium pyruvate (1,2). It was noted that a commercial preparation of sodium pyruvate did not have the favorable effect obtained with sodium pyruvate prepared in the laboratory(3). This fact, combined with the recent isolation by Montgomery and Webb(4) of an impurity present in some samples of sodium pyruvate, which inhibited the tricarboxylic acid cycle in isolated mitochondria, suggested an investigation of the possible antimalarial effects of this impurity. Two samples of this material, identified as a lactone of parapyruvic acid (the dimer of pyruvic acid), have been obtained through the kindness of Dr. Carmel Montgomery. They have been tested against *P. lophurae* maintained *in vitro* both extracellularly and intracellularly, as well as against the infection *in vivo.*

Materials and methods. The lactone of parapyruvic acid was dissolved in water (redistilled in a Pyrex glass still), and the solution was brought to pH 6.8 with 1 N NaOH and diluted to give a concentration of 15 mg/ml. The solution was sterilized by filtration through an ultrafine glass filter, tubed in small amounts, frozen and stored at −20°C. Freshly thawed material was appropriately diluted and used for the experiments. Since it was added to cultures having a pH of 6.8 which were subsequently held at least 1 day at 40°C, it is probable that the lactone was soon converted to parapyruvate, the form found by Montgomery and Webb to be effective in inhibiting mitochondrial respiration. For experiments with *extracellular* parasites, blood from a duckling heavily infected with *P. lophurae* was treated with guinea pig complement and an anti-duck erythrocyte serum prepared in rabbits(2). This resulted in a mixture containing free parasites, free red cell nuclei and clumps of agglutinated, mostly hemolyzed erythrocytes, both with and without parasites. A purified suspension containing only free parasites and free red cell nuclei was obtained by removing the agglutinated cells by means of a 40-second low speed centrifugation (to a maximum speed of 400 rpm). The parasite suspension was added, in amounts of 0.6 ml for purified suspensions and 0.3 ml for unfractionated suspensions, to 50 ml flasks lined with a thin plasma clot(3) and containing 3.3 ml of culture medium. The medium was a concentrated extract of duck erythrocytes prepared in the manner previously described(1) and containing all of the supplements of known chemical nature which have been found(5) to favor the extracellular survival of these intracellular parasites (yeast adenylic acid, coenzyme I, ATP, coenzyme A, malate and pyruvate). Appropriate small volumes of suitable dilutions of the stock solution of parapyruvate were added to some of the cultures. The flasks were incubated on a rocker at 40°C, and a current of air with 5% CO_2 was passed through them.

TABLE I. Effect of Parapyruvate on Extracellular Survival *in vitro* of *Plasmodium lophurae*. Culture medium was a duck erythrocyte extract prepared as previously described, except in Exp. A where the extract was diluted with isotonic diluent to one-third its usual strength. A purified suspension of extracellular parasites served as inoculum in Exp. A-D. In Exp. E an unpurified mixture of hemolyzed, agglutinated erythrocytes and free parasites was used.

Exp.	Flasks	Parapyruvate added, mg/ml	% degenerate parasites in stained films made after 20 hr at 40°C
A	1, 2	0	3, 2
	3, 4	.85	32, 25
B	1, 2	0	3, 2
	3, 4	.40	14, 15
C	1, 2	0	10, 9
	3, 4	.40	20, 20
	5, 6	.04	20, 23
	7, 8	.008	12, 19
D	1	0	1
	2	.4	13
	3	.04	6
	4	.008	1
E	1, 2	0	1, 1
	3, 4	.08	4, 5
	5, 6	.025	7, 4
	7, 8	.008	2, 3

An initial Giemsa-stained film was prepared from the parasite suspension used as inoculum. After one day of incubation samples were taken from each flask and used for the preparation of Giemsa-stained films and wet mounts. The latter were examined with a phase contrast microscope(1). The stained films served for counts of the proportions of degenerate(1) parasites. For experiments with parasites developing *intracellularly* in suspensions of intact duck erythrocytes the medium and methods were those previously found to support multiplication of *P. lophurae*(6,7). Each culture flask held 6 ml of a suspension containing about 1,000,000 red cells per cu mm, with about 50 parasites per 1000 red cells. The parapyruvate was again added as small volumes of appropriate dilutions of the stock solution. The flasks were incubated on a rocker at 40°C, and a current of air with 5% CO_2 was passed through them. Giemsa-stained films were prepared from each flask initially (Day 0) and after 1 and 2 days of incubation. These were used to count the parasites per 1000 red blood cells and the proportions of parasites in different stages of development.

Results. The deleterious effect of the parapyruvate preparation on the extracellular survival of *P. lophurae* is clearly shown by the

TABLE II. Effect of Parapyruvate on Development of *P. lophurae* in Suspensions of Duck Erythrocytes Maintained *in vitro*, in 8 Flasks.

Parapyruvate added, mg/ml	% young parasites (uninucleate, not pigmented) on day: 0	1	2	Total parasites/1000 red blood cells on day: 0	1	2
0	48	0	68	50	52	59
0	74	0	68	52	51	51
.075	52	0	24	52	50	18
.075	64	0	36	48	33	29
.025	48	2	60	48	42	20
.025	56	0	64	41	31	21
.008	72	0	68	50	43	26
.008	80	0	74	68	82	20

experiments summarized in Table I. Concentrations as low as 40 and 25 μg per ml were definitely harmful to the parasite, whereas a concentration of 8 μg per ml appeared to be at the border line of activity. The relative degrees of degeneration seen in the stained films were likewise seen in the fresh preparations examined by phase contrast.

The parapyruvate similarly had a deleterious effect on the development of the parasites within erythrocytes, as illustrated by the experiment shown in Table II. It will be seen that all 3 concentrations tested reduced the number of parasites present after 2 days' incubation to about half that of the control flasks. Some reduction in parasite numbers was already evident at the two higher concentrations after one day's incubation. It is worth noting that in the flasks with the highest concentration (75 μg/ml) the proportion of young parasites which reinvaded erythrocytes on day 2 was much less than in the control flasks or in those with lower concentrations.

Discussion. The inhibition of respiration of isolated mitochondria by parapyruvate appears to depend on competition between this compound and alpha-ketoglutarate for alpha-ketoglutaric acid oxidase(4). Since there is good evidence for the participation of the tricarboxylic acid cycle in the oxidative metabolism of malaria parasites(8), it may well be that the deleterious effect of parapyruvate on the parasites is likewise a result of interference by this compound with the utilization of alpha-ketoglutarate. In the experiments with extracellular parasites this effect occurred at concentrations down to 1.5 x 10^{-4}M in the presence of pyruvate at a concentration of 5 x 10^{-3}M. In the experiments with parasites developing intracellularly the effect was marked even at the lowest concentration tested of 5 x 10^{-5}M. Perhaps the absence of added pyruvate and the longer period of incubation in the latter experiments both contributed to this greater effect.

The ability of the parapyruvate to penetrate infected duck erythrocytes and reduce the numbers of *P. lophurae* developing intracellularly suggested that the compound might have some antimalarial action *in vivo*. This did not prove to be the case. Ducklings infected with *P. lophurae* were treated once on the 3rd day and twice on each of the 4th and 5th days of their infection with doses of 2.5 to 10 mg parapyruvate per 100 g body weight, administered in solution intravenously. No effect on either the course of the infection or the ducklings was observed. Presumably the parapyruvate was altered before it could exert its action on the malaria parasites.

Summary. When the erythrocytic forms of *Plasmodium lophurae* were maintained extracellularly *in vitro* the addition of parapyruvate at concentrations down to 25 μg per ml increased the proportion of degenerate parasites present after one day's incubation. In cultures with intracellular parasites in suspensions of intact duck erythrocytes, the addition of the parapyruvate at concentrations down to 8 μg per ml decreased sharply the numbers of parasites present after two days' incubation. Somewhat higher concentrations also interfered with the reinvasion of erythrocytes by merozoites formed in such cultures. No antimalarial effect was observed when the compound was administered intravenously to ducklings infected with *P. lophurae*.

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Paradoxical Retention of Uric Acid by Uricosuric Drugs in Low Dosage.* (22094)

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It has long been known that the renal excretion of uric acid in man is increased by large doses of salicylate and suppressed by small doses(1,2). Recent, more adequately documented studies(3) showed that acetylsalicylic acid in 5.2 g daily oral dosage caused a mean increase of 37.2% in the 24-hour urinary uric acid output of 15 subjects, with a concomitant mean fall of 25.5% in plasma uric acid, whereas 2.0 g daily dosage resulted in a mean decline of 14.4% in the urinary excretion and 7.5% rise in the plasma concentration of uric acid. Renal clearance studies indicate that this reversible effect is related to the concentration of salicylate in blood plasma(4) or, more precisely, in the urine(3); high salicylate concentrations cause an increase in the renal clearance ratio, $C_{\text{uric acid}}/C_{\text{inulin}}$, low salicylate levels depress this ratio.

Our data illustrate these relationships in respect to salicylate and indicate further that the reversibility of effect on uric acid clearance can be demonstrated, by appropriately designed experiments, to occur with all other uricosuric drugs we have examined, namely, phenylbutazone, phenylbutazone analogs, and probenecid. It would appear that the capacity to cause uric acid retention when given in low dosage is, to greater or lesser degree, a property common to uricosuric agents. The observation may have implications of interest in relation to the still obscure nature of renal tubular transport systems for uric acid.

Methods. The experiments were performed in gouty subjects in the intercritical phase of the disorder. Standard renal clearance technics(5) were employed throughout, with minor modifications described elsewhere(6). The general experimental design was to obtain 3 or 4 pre-medication urine collection periods for estimation of the inulin and PAH clearances, then to infuse the drug to be studied at a slow rate yielding low initial plasma drug concentrations, and gradually to increase the rate of drug infusion to attain uricosuric plasma drug levels. The 6-10 urine collection (catheterization) periods obtained during infusion of the drug in each experiment were each of 10-30 minutes duration; urine samples (spontaneous voidings) collected in some instances after termination of the drug infusion represented 2-hour intervals during the day and 4-hour intervals at night. Blood samples were procured at the midpoint of each urine collection period. In the salicylate infusion experiments, 4 g sodium salicylate dissolved in 500 ml isotonic saline solution was injected at a rate of 12-15 mg salicylate/min. for one or more periods, with an increment of 5-10 mg/min. in each urine collection period thereafter. In 2 phenylbutazone experiments, the infusing solution contained 2 g phenylbutazone/250 ml isotonic saline solution, was delivered at a rate of 12 mg/min. for 2 clearance periods and at double the rate thereafter. In the 3 remaining phenylbutazone, the 2 G-25671 and 6 of the 8 phenylbut-

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TABLE I. Representative Experiments Showing Effect of Slow Infusion of Sodium Salicylate, Phenylbutazone and Probenecid on Uric Acid Clearance.

Case	Period (min.)	Rate of drug infusion (mg/min.)	Plasma drug conc. (mg %)	Plasma uric acid (mg %)	$C_{\text{uric acid}}$ (ml/min.)	$\frac{C_{\text{uric acid}}}{C_{\text{inulin}}}$ (%)
J. S.	Control	0	0	11.5	13.9	10.7
	"	0	0	11.5	14.9	11.7
	"	0	0	11.5	14.4	11.2
		Sodium salicylate infusion (4 g/500 ml saline sol.)				
	15	12	Trace	11.4	13.9	10.7
	13	12	1.5	11.3	7.95	6.36
	11	17	3.1	10.9	7.08	5.66
	11	20	5.8	10.8	9.58	7.92
	12	25	9.0	10.9	13.4	11.4
	12	29	12.0	10.9	18.0	14.4
	12	34	15.0	10.8	21.6	18.6
	10	40	17.1	10.6	27.2	23.6
	11	45	21.0	10.7	31.5	26.5
L. R.	Control	0	0	9.1	8.05	8.47
	"	0	0	9.1	8.09	8.00
	"	0	0	9.2	8.02	8.41
		Phenylbutazone infusion (2 g/250 ml saline sol.)				
	15	12	2.3	9.5	6.15	6.73
	16	12	3.0	9.4	6.04	6.65
	15	24	10.0	9.5	10.4	10.6
	15	24	12.0	9.5	12.7	14.3
	15	24	15.5	9.3	13.9	18.0
	10	24	16.7	9.0	24.4	23.4
R. G.	Control	0	0	7.3	8.73	6.35
	"	0	0	7.2	9.14	5.53
	"	0	0	7.0	8.07	6.52
		Probenecid infusion (0.1 g/500 ml 5% glucose sol.)				
	15	.2		7.4	8.07	5.08
	15	.2		7.6	5.46	4.48
	19	.2		7.2	6.15	4.70
	16	.4		7.0	7.17	5.48
	16	.4		6.9	7.63	5.26
	13	.4		7.0	8.03	5.11
	19	.6		7.1	7.42	5.30
	20	.6		7.0	8.45	6.12
	23	1.2		7.0	10.6	6.80
	17	1.2		7.0	12.0	8.17

azone metabolite 1 experiments, the initial infusion rate was 1.4 mg/min. with an increase of 1.4-2.0 mg/min. in each following collection period. In 2 of the metabolite 1 experiments cited the drug was infused rapidly in a total dosage of 8 mg/kg body weight. The details of the probenecid injections are given in the text. Uric acid was determined by a modification of the method of Buchanan *et al.* (7) incorporating the use of uricase, urea cyanide-carbonate and arseno-phosphotungstic acid, inulin by the Schreiner method(8), creatinine by the method of Bonsnes and Taussky(9), salicylate by the method of Brodie *et al.*(10). For the plasma phenylbutazone, G-25671 and metabolite 1 determinations we are indebted to Dr. J. J. Burns.

Results. Salicylate. Rapid infusion of sodium salicylate at a rate sufficient to maintain plasma total salicylate concentrations at 15-34 mg % effected an increase in $C_{\text{uric acid}}/C_{\text{inulin}}$ from a mean of 5.5% in the control clearance periods to a mean of 20.6% in 6 subjects so studied(3). The experiment included in Table I (Case J.S.) illustrates the effects on $C_{\text{uric acid}}/C_{\text{inulin}}$ of a slow, sustained infusion of sodium salicylate delivered at a rate permitting gradual increase of plasma total salicylate from trace levels to 21 mg %. In the 3 control periods, $C_{\text{uric acid}}/C_{\text{inulin}}$ varied from 10.7 to 11.7%, and remained within this range in the initial period of the infusion. At a

TABLE II. Summary of Slow Infusion Experiments with Uricosuric Drugs, Showing Maximal Suppression of Uric Acid Clearance at Low Plasma Drug Concentrations.

				$C_{\text{uric acid}}/C_{\text{inulin}}$		
	Case	Drug infused	Plasma drug conc. (mg %)	Control (%)	Minimum (%)	Change (%)
1.	M. L.	Salicylate	2.2	7.22 ± .29	3.38	−53.2
2.	J. S.		2.8	11.2 ± .41	5.66	−49.5
3.	S. K.		4.7	5.14 ± .14	2.80	−45.6
4.	M. M.		1.8	5.48 ± .33	3.10	−43.5
5.	M. S.		4.1	6.40 ± .72	4.11	−35.8
6.	J. B.		8.4	5.16 ± .21	4.38	−15.8
1.	L. O.	Phenylbutazone	2.1	5.73 ± .15	3.48	−39.3
2.	N. P.		4.0	1.87 ± .24	1.22	−34.8
3.	J. S.		2.6	5.24 ± .55	4.20	−19.8
4.	L. R.		3.0	8.30 ± .46	6.65	−18.9
5.	J. B.		1.3	5.15 ± .29	4.72	+ 8.4
1.	M. G.	Metabolite 1	.5	4.97 ± .31	3.34	−32.8
2.	J. Z.		3.6	4.72 ± .25	3.22	−31.8
3.	G. H.		7.0	3.79 ± .13	2.85	−24.8
4.	M. R.		5.9	6.60 ± .17	5.15	−22.0
5.	H. B.		4.3	4.92 ± .35	3.90	−20.7
6.	J. B.		1.3	8.94 ± .14	6.99	−20.7
7.	N. K.		5.0	3.72 ± .33	3.05	−18.0
8.	I. S.		.4	6.98 ± .17	7.67	+10.0

plasma total salicylate concentration of 1.5 mg % $C_{\text{uric acid}}/C_{\text{inulin}}$ was depressed to 6.36%, and persisted well below the premedication ratio until the plasma total salicylate reached 9 mg %, at which point $C_{\text{uric acid}}/C_{\text{inulin}}$ returned to the control limits. At plasma total salicylate levels of 12 mg %, $C_{\text{uric acid}}/C_{\text{inulin}}$ exceeded the premedication ratios and continued to rise with further augmentation of the plasma drug concentration, ultimately reaching 26.5% at a plasma total salicylate level of 21 mg %. In Table II, the pertinent results of 6 such salicylate infusion experiments are recorded. It will be noted that a (transitory) decline in $C_{\text{uric acid}}/C_{\text{inulin}}$ occurred in each instance, the peak decline varying from 53.2-15.8% of the mean premedication figures. In five of these subjects the decline exceeded 30%.

Fig. 1 illustrates an experiment (Case M.L.) in which the plasma total salicylate again was slowly raised by infusion of sodium salicylate, the infusion was then discontinued, and the plasma drug concentration decay curve followed. It will be noted that $C_{\text{uric acid}}/C_{\text{inulin}}$ was depressed at low plasma total salicylate levels in both the initial ascending and terminal descending segments of the curve representing plasma drug concentrations. Distinct uricosuria occurred in the intervening period of plasma total salicylate levels in excess of 10 mg %.

Phenylbutazone. Rapid infusion of phenylbutazone in 10 subjects consistently caused a rise in $C_{\text{uric acid}}/C_{\text{inulin}}$, from a mean control value of 6.0% to a mean peak of 19.5%(11). The effects of a slow, sustained infusion are illustrated in Table I (Case L.R.). $C_{\text{uric acid}}/C_{\text{inulin}}$ fell initially, from a mean control figure of 8.3% to 6.7% at low plasma phenylbutazone concentrations, then rose to uricosuric levels as the plasma drug concentration exceeded 10 mg %. The results of 5 such experiments are summarized in Table II; in 4 a transitory decline in $C_{\text{uric acid}}/C_{\text{inulin}}$ was detected, the peak decline in 2 instances exceeding 30%. Unlike the experience with salicylate, observations for 24-72 hours following termination of infusion of phenylbutazone(11) failed to reveal any post-medication suppression of $C_{\text{uric acid}}/C_{\text{inulin}}$. This is attributed to the slow rate of biotransformation of phenylbutazone (approximately 20%/day)(12) and the consequent persistence of high plasma and urinary drug concentrations(11).

Phenylbutazone analogs. Phenylbutazone metabolite 1 (p-hydroxyphenylbutazone) (13), which exhibits uricosuric activity approximating that of phenylbutazone in equiv-

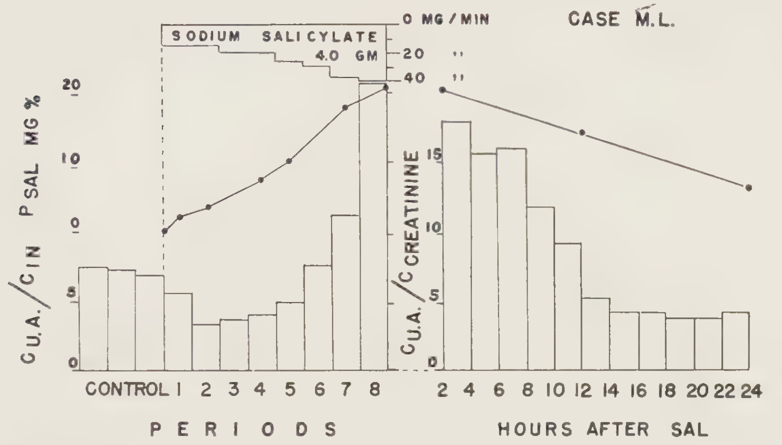

FIG. 1. After 3 control periods, sodium salicylate was infused slowly and plasma total salicylate level gradually raised (linear curve, top). Infusion was discontinued after eight 20-min. periods. Effects of rising and falling plasma drug levels on $C_{\text{uric acid}}/C_{\text{inulin}}$, respectively, $C_{\text{uric acid}}/C_{\text{creatinine}}$ are indicated by the bars (see text).

alent dosage, at low plasma drug levels caused a transitory decline in $C_{\text{uric acid}}/C_{\text{inulin}}$ in 7 of 8 experiments (Table II). In 2 instances the decline exceeded 30%. G-25671, a phenylbutazone derivative in which the butyl side chain is replaced by a phenylthioethyl group, is a more potent uricosuric agent(12). In 7 rapid infusion experiments, G-25671 caused a mean 5-fold increase in $C_{\text{uric acid}}/C_{\text{inulin}}$, from a mean pre-medication value of 6.15% to a mean peak of 30.6%(14). In 2 slow infusion experiments, a transient, moderate decline in $C_{\text{uric acid}}/C_{\text{inulin}}$, reaching –16.1% at its peak, was observed at a plasma G-25671 level of 0.6 mg %; at plasma drug levels of 1.0 mg % uricosuria was already in evidence.

Probenecid. Suppression of $C_{\text{uric acid}}/C_{\text{inulin}}$ could be demonstrated with this potent uricosuric agent(6) only by slow infusion of very small quantities of the drug. Injection at the rate of 0.2 mg probenecid/min. gave a maximum decline in $C_{\text{uric acid}}/C_{\text{inulin}}$ of 27% below the mean ratio of the control period (Case R.G., Table I); at 0.3 mg probenecid/min., the maximum decline was 14.6%; at 0.4 mg/min. suppression of uric acid clearance was no longer demonstrable; and at higher drug infusion rates uricosuria set in promptly.

Discussion. The observations herein recorded may explain, in part, some of the conflicting results reported in the literature, notably in respect to phenylbutazone. This drug has been found by most investigators to increase urinary excretion of uric acid in ordinary therapeutic dosages by mouth, but by others to have no effect or, indeed, to cause retention of uric acid. This confusion is compounded by the fact that phenylbutazone consistently lowers the serum uric acid level even in instances without uricosuria. This discrepancy has been interpreted to imply suppression of uric acid formation by phenylbutazone but direct measurement of the rate of uric acid biosynthesis, using N^{15}-labeled glycine in subjects receiving the drug, fails to support this view(15). The evidence now available indicates that such discrepant declines in serum uric acid are apt to be factitious, reflecting hemodilution due to renal retention of sodium and water caused by phenylbutazone(11,14). Further clarification of this relationship has been achieved by renal clearance studies, including those reported here, which reveal the sequence of renal effects at rising plasma phenylbutazone levels, corresponding to various dosages of phenylbutazone. Appreciable retention of salt and water may begin in association with plasma phenylbutazone levels as low as 1 mg % and continues at higher drug levels(11,14); suppression of uric acid clearance occurs within an intermediate range of plasma phenylbutazone concentration; and distinct uricosuria begins at plasma phenylbutazone concentrations of about 10 mg %, becoming more marked at higher drug levels(11,14,16). These observations explain how, without suppression of uric acid biosynthesis, administration of phenylbutazone may cause lowering of the serum uric acid level without uricosuria, indeed in the face of uric acid retention.

Discontinuance of salicylate, probenecid and other uricosuric drugs is usually followed by one or 2 days of uric acid retention. This phenomenon, hitherto regarded as "compensatory," would now appear to reflect decline of the plasma drug concentration to the low levels associated with depression of the uric acid clearance. In this connection it should be pointed out that the concentration of drug in the tubular urine is probably more critical than that in plasma, in respect to the reversible action on $C_{\text{uric acid}}/C_{\text{inulin}}$. To be sure, in ordinary circumstances, as in the experiments here recorded, the urinary drug concentration may be presumed to be a function of the plasma drug level but when the two are dissociated—as by concomitant administration of salicylate and sodium bicarbonate—it is found that the determining factor in regulating uric acid clearance is the drug concentration in urine(3,17). The point may be significant in respect to the immediate mechanisms involved.

The uricosuric effects of salicylate, probenecid, phenylbutazone and phenylbutazone derivatives are all ascribable to suppression of tubular reabsorption of uric acid, since clearance studies uniformly show an associated distinct *rise* in $C_{\text{urate}}/C_{\text{inulin}}$ in each instance(4,6,11,14). Conversely, the clearance data of this paper reveal that the uric acid retention produced by these same drugs in low dosage is uniformly referable to a distinct *fall* in $C_{\text{urate}}/C_{\text{inulin}}$. This paradoxical reversibility of effects presumably is related to some characteristic of the tubular transport systems for uric acid, since there is no indication of significant changes in filtered load, *i.e.*, in the filtrability of plasma uric acid(18) or in the glomerular filtration rate. The nature of this characteristic must, for the present, be a matter of speculation. It has already been suggested that a single system for tubular reabsorption of uric acid, presumed to be enzymatic, may be stimulated by low concentrations and inhibited by high concentrations of the same drug(4). Another possibility, not excluded by the evidence at hand, is that 2 more or less distinct systems of tubular transport of uric acid are involved. One of these, of very limited capacity, might be concerned with tubular excretion of uric acid and inhibited by low (and high) drug concentrations; the other, of considerably greater capacity, is clearly responsible for tubular reabsorption of uric acid and evidently is suppressed only by high drug concentrations.

Summary. Salicylate, phenylbutazone, certain phenylbutazone derivatives and probenecid, all of which in high plasma drug concentrations increase the uric acid clearance, are demonstrated by appropriate renal clearance technics to depress the uric acid clearance at low plasma drug concentrations. The significance of this paradoxical reversibility of effects is discussed in relation to controversial aspects of phenylbutazone uricosuria, the phenomenon of "compensatory" uric acid retention after discontinuance of uricosuric agents, and the nature of tubular transport mechanisms for uric acid.

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Hemolytic Mechanism in Sickle Cell-Hgb C Disease.* (22095)

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Splenic overactivity has been implicated in the hemolysis observed in many of the acquired hemolytic syndromes. The role of the spleen in the red cell destruction characteristic of the hereditary hemolytic anemias, however, has not been clearly defined. The available data suggest basically different mechanisms for the pathogenesis of the increased hemolysis in 2 of the hereditary hemolytic syndromes. In sickle cell anemia (homozygous Hgb S disease) the frequent development of an atrophic spleen, the so-called autosplenectomy, implies that the spleen cannot play an active role in red cell destruction. Furthermore, splenectomy in cases of uncomplicated sickle cell anemia neither leads to hematologic improvement nor results in a prolongation of the lifespan of the erythrocyte. Thus, sickle cell anemia erythrocytes transfused into a splenectomized sickle cell anemia patient still showed a markedly reduced lifespan(1). In hereditary spherocytosis, however, the evidence suggests that the increased destruction of the red cells may be mediated through the presence of a normal spleen, since hereditary spherocytosis erythrocytes transfused into a normal individual lacking a spleen have a normal survival time whereas the same cells given to a normal individual with a spleen demonstrate a sharply reduced lifespan(2). This situation exists irrespective of the pre- or post-splenectomy state of the donor with hereditary spherocytosis, or his hematologic status at the time of study. In view of these considerations, it seemed of some interest to perform similar studies on patients with sickle cell-Hgb C disease. Sickle cell-Hgb C disease is characterized by splenomegaly and signs of hemolysis which appear to vary in degree intermittently during the course of the disease. Because splenomegaly often becomes evident at times of hemolytic crises in sickle cell-Hgb C disease, there is reason to suspect that enlargement of the spleen may lead to accelerated rates of red cell destruction. By defining the part played by the spleen in the hemolysis of this disease, it was hoped that an evaluation of the effects of splenectomy and the possible use of this operation as a therapeutic procedure might thereby be made.

Material and methods. Three patients with the diagnosis of sickle cell-Hgb C disease, confirmed by electrophoretic analysis of their hemoglobin types, were used as donors. All were in a relatively asymptomatic state. The spleens of 2 of the donors were palpable just below the costal margin. From one patient, A. F., two 500 ml aliquots of blood were obtained and transfused into a normal individual with a spleen and into a hematologically normal splenectomized recipient who had

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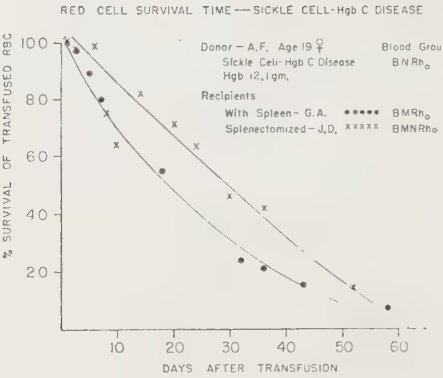

FIG. 1.

been subjected to surgery some years earlier for idiopathic thrombocytopenic purpura. From the second patient, I. F., the younger sister of A. F., 500 ml of blood were removed and transfused into a second splenectomized recipient. The survival time of the erythrocytes was followed by the Ashby technic of differential agglutination in the standard way using dried anti-M serum which gave inagglutinable counts of less than 10,000 cells per cu mm(1). The third donor with sickle cell-Hgb C disease, L. M. (blood type O N Rh$_0$) was utilized for a combined Ashby and Cr^{51} tagged red cell survival time study. Approximately 500 ml of blood were removed by venesection and three 20 ml aliquots of blood were tagged with 75 microcuries of Cr^{51} by a modification of the method of Weinstein, *et al.*(3). One aliquot of the Cr^{51} tagged cells was auto-transfused into the donor, a second given to a compatible normal recipient (blood type B M Rh$_0$), the third to a splenectomized normal individual of blood type AB MN Rh$_0$. The remainder of the blood was transfused into the normal recipient with a spleen who had been given the tagged cells. The disappearance of the Cr^{51} from the peripheral blood, determined at intervals of one or 2 days, was carried out in a well-type scintillation counter and the half-time disappearance (t/2) calculated(3). The Ashby technic was used to follow the survival of the transfused red cells in the normal recipient.

Results and discussion. In the first experiment, performed by the Ashby technic alone, no appreciable difference was noted between the survival times of red cells from the patient with sickle cell-Hgb C disease transfused into the normal and splenectomized individuals. In each instance the red cells disappeared from the recipient's circulation in approximately 50 to 60 days (Fig. 1). Similar data were obtained in the second experiment in which sickle cell-Hgb C disease erythrocytes were transfused into a splenectomized recipient. The red cell lifespan was again approximately 60 days (Fig. 2). Since no normal control was run at the same time, it is impossible to say whether this result represents a prolongation of the erythrocyte lifespan in the splenectomized subject. However, the similarity of the findings in each of the first 2 experiments suggests that any increase in red cell survival time in the splenectomized recipient must have been slight, if at all present. In the third experiment, the half-time of disappearance of Cr^{51} tagged

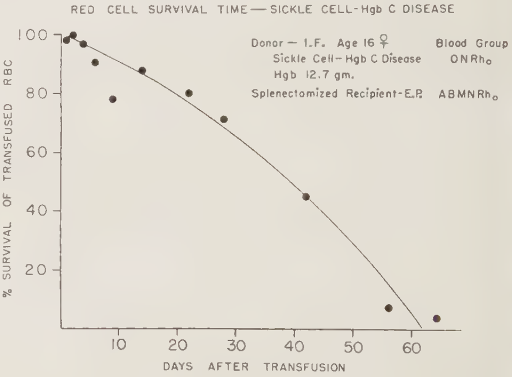

FIG. 2.

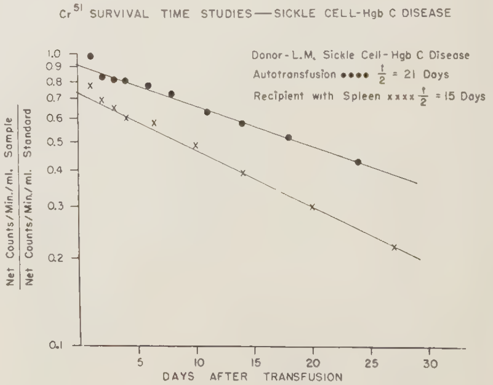

FIG. 3.

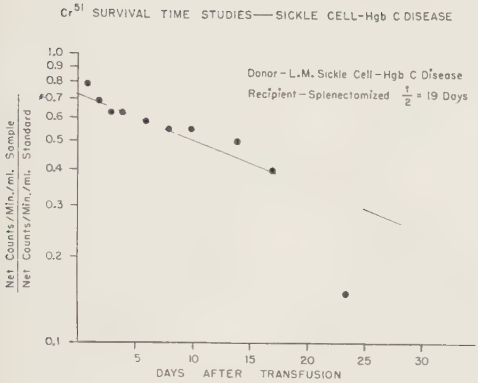

FIG. 4.

cells transfused back into the donor was 21 days, while in a normal individual these cells had a t/2 of 15 days (Fig. 3). The aliquot of labelled sickle cell-Hgb C disease erythrocytes followed in the splenectomized recipient appeared to have a t/2 of approximately 19 days, although a single satisfactory line of disappearance for the Cr^{51} could not be drawn (Fig. 4). The configuration of the curve suggested that 2 or more populations of red cells were present or that the Cr^{51} was being eluted from the pathologic cells at an inconstant rate. Nevertheless, the major proportion of the Cr^{51} disappeared at approximately the same rate in the splenectomized and non-splenectomized recipients. The erythrocyte lifespan by the Ashby technic was, as in the instance of the first 2 donors, approximately 50 days in the normal recipient (Fig. 5).

These observations suggest that the intrinsic defect of sickle cell-Hgb C erythrocytes, like that of sickle cell anemia red cells, is primarily responsible for the shortened survival time of the red cells. As in sickle cell anemia, hemolysis proceeds at approximately the same rate whether a spleen is or is not present. The pathogenesis of the hemolytic mechanism is, therefore, somewhat different from that seen in hereditary spherocytosis where the presence of a normal spleen is required for red cell destruction to take place at an increased rate. These studies do not throw any light on the site of red cell destruction in sickle cell-Hgb C disease. It has been postulated that in sickle cell anemia, by virtue of the abnormal shape of the red cells, plugging of small vessels with resultant stasis and hemolysis takes place. Since this phenomenon may occur in any small vessel suitably situated, the spleen does not selectively trap such cells. In hereditary spherocytosis, the spherical erythrocytes are believed to be selectively trapped in the sinusoids of that organ. It would seem, therefore, that the sickle-shaped erythrocytes in sickle cell-Hgb C disease behave much like the red cells in the related homozygous Hgb S condition.

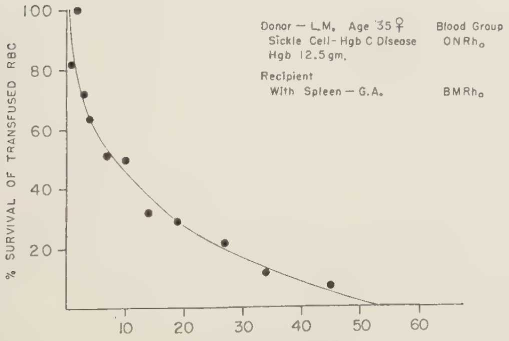

FIG. 5.

In vivo sickling can be correlated with the percentage of the total hemoglobin in the form of Hgb S(4,5). Since the majority of patients with sickle cell anemia have approximately 90% Hgb S(6,7) while those with sickle cell-Hgb C disease have from 45-50% Hgb S(8), it would be expected that under similar conditions hemolysis as a consequence of the mechanical factors mentioned above would take place more readily in sickle cell anemia. Furthermore, since some patients with homozygous Hgb S disease have as little as 60% Hgb S(9) while some with sickle cell-Hgb C disease have over 63% Hgb S(10), some overlap in the severity of the anemia and degree of hemolysis should be encountered if red cell destruction in these sickling diseases is to be explained on the basis of mechanical trapping, stagnation and lysis. Clinical and hematologic data in the 2 sickle cell diseases are consistent with these tenets and support this concept. It would be of extreme interest to try to localize if possible major sites of stasis and destruction in these sickling disorders. Unfortunately, body scanning following the injection of Cr^{51} tagged cells was not possible, but such studies would undoubtedly provide important relevant information.

Another problem left unanswered by the present investigation is the role of the spleen during periods of clinical crisis. Characteristic of the crisis of sickle cell-Hgb C disease is the progressive development of marked anemia and a rapidly enlarging spleen. The clinical findings are, therefore, in marked contrast to those observed in the usual variety of crisis seen in sickle cell anemia, where the abdominal, muscle and joint pains are not accompanied by any specific hematologic changes nor any increase in the degree of hemolysis(11). Abdominal pain in sickle cell-Hgb C disease is usually localized in the left upper quadrant and can be correlated with the remarkable change in the size of the spleen. It seems more than likely that splenic overactivity, either by virtue of its increasing mass, or as a result of a more specific role in sequestering the abnormally shaped red cells, leads to the development of the severe anemia seen during periods of crisis in sickle cell-Hgb C disease. Furthermore, the number of patients with sickle cell-Hgb C disease who appear to develop the first episode of anemia following trauma to the left upper quadrant area strongly suggests that splenic enlargement may actually be the precipitating factor in the pathogenesis of the crisis, while the anemia is a secondary phenomenon. Unfortunately none of our patients was suffering from this complication at the time of observation, but repeat Cr^{51} auto-survival time studies of the erythrocytes during periods of quiescence and crisis will make it possible to evaluate this problem.

Summary. Red cells from 3 patients with sickle cell-Hgb C disease transfused into normal individuals with and without a spleen, were found to have a survival time of approximately 50 days. The spleen, therefore, does not appear to have a critical role in the hemolysis occurring in this disease. Possible mechanisms for the red cell destruction, based on the per cent of Hgb S present, as well as the role of the spleen during periods of crisis, are discussed.

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Hyperactivity of Metachloridine against *Plasmodium gallinaceum* in Chicks Maintained on a Purified Diet.* (22096)

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In a previous report(1), it was shown that sporozoite-induced *Plasmodium gallinaceum* infections behave similarly in chicks fed a purified diet or a commercial stock diet. It was thought, however, that the effect of therapeutic agents might differ in chicks fed different dietary regimens, since several antimalarials are known to be, or are suspected of being, antimetabolites. Commercial stock diets are known to vary in their composition from time to time and conceivably could vary in their content of critical natural components which might inhibit the activity of certain antimalarials. The variability in the content of diet of such natural metabolites might account for the minor variations occasionally encountered in the activity of antimalarials. Alternatively, the presence of antibiotics currently being added to stock feed might have some influence on the results of tests with antimalarials.

We undertook to determine the effect of a purified diet (C-2(1,2)) on the parasitemia produced by *P. gallinaceum* in untreated chicks after the intravenous inoculation of erythrocytic parasites and the effect of such a diet on the antimalarial activity of certain compounds. As will be shown, the use of the purified diet had little or no effect on the antimalarial activity of most of the compounds tested. However, metachloridine (2-metanilamido-5-chloropyrimidine) and sulfadiazine were strikingly more active in chicks fed the purified diet than in those fed the stock diet. The hyperactivity of sulfadiazine might have been the result of the absence of *p*-aminobenzoic acid from the purified diet; however, neither *p*-aminobenzoic nor *m*-aminobenzoic acid had any effect on the antimalarial activity of metachloridine(3), nor, to date, has any natural substance been isolated which can reverse the activity of metachloridine. This drug was placed by Josephson *et al.*(4) in a class of antimalarials characterized as plasmodistatic, acting only during the dividing stages of the parasite. It was predicted that the parasite could be made resistant to members of this class of antimalarials, and Bishop and McConnachie(5) found that resistance to metachloridine can be readily induced. Josephson *et al.*(4) predicted that members of this class would be antimetabolites. The fact that metachloridine was more active in chicks fed the purified diet than in those fed stock diet suggested that the latter diet contained an inhibitor, possibly a substance essential for the parasite. A search for such a substance was undertaken, and the results are reported in this paper.

Methods. New Hampshire chicks from a single source were used throughout the experiments. They were received in the laboratory when one day old, separated into 2 groups, and each group immediately placed on one of the test diets, on which they continued until the end of the experimental period. Weights of the chicks were recorded after they had been on the diets for 7 days and again 4 days later. The drug tests were conducted according to a procedure previously described(6). Week-old chicks were inoculated intravenously with 16×10^6 erythrocytes parasitized with *P. gallinaceum*. Parasite counts (parasitized erythrocytes per 10^4 erythrocytes) were made on the morning of day 4 (day of inoculation is day 0). Drugs were administered orally, twice daily for 4 days, beginning 4 to 5 hours before parasite

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* The authors are grateful to the following: the Archer-Daniels-Midland Co. for furnishing the soy grits and hulls; the Soya Products Division of the Glidden Co. for the soy granules; Drs. M. R. Spivey Fox and G. M. Briggs for helpful suggestions; Anne Jowett, G. W. Rusten, and J. Phillips for technical assistance.

TABLE I. Comparison of Antimalarial Activity of 9 Compounds Tested in Chicks Infected with *P. gallinaceum* and Fed Either the C-2 Diet or the Stock Diet.

Compound	Minimum effective dosage* (mg/g body wt) C-2 diet	Stock diet
Quinine	.005	.01
Chloroquine	.001	.002
Atabrine	.005	.01
Chlorguanide	.0005	.002
	.002	.002
Terramycin	.04	.08
Fumagillin	>.04	>.04
Pamaquine	.0003125	<.000625
Sulfadiazine	<.025	.1
	≤.003125	.05
Metachloridine	.000125	.002
	.0000625	.002
	<.0000625	.0005

* Each result derived from one experiment.

inoculation. The stock diet used was Purina Startena; the purified diet was prepared in this laboratory according to published procedures(1,2). Henceforth, the purified diet will be termed the C-2 diet. Variations in the C-2 diet were made by replacing a portion of the Cerelose with the test quantity of the nutrient to be studied. In the experiments designed to test the effect of nutrient variables of the C-2 diet on the antimalarial activity of metachloridine, the drug was given at 5 levels to those chicks given the varied C-2 diet and at 3 levels to those fed the stock diet. In late experiments, the C-2 diet served as the control diet. The corn-soy diet (a complete all-vegetable diet)(7) contained the following constituents per kilo of diet: 575 g of ground, whole, yellow corn; 350 g of soybean meal; 10 g of Cerelose; 5 g of corn oil; 60 g of Briggs chick salts-A(2); 0.02 mg of vit. D_3, 8 mg of riboflavin; and 0.02 mg of vit. B_{12}.

Results. The mean 4-day weight gain of 60 parasitized chicks in 12 series fed stock diet was 20.1 ± 0.6 g; the corresponding 60 chicks fed the C-2 diet gained 18.5 ± 0.9 g. The difference between the means was not significant ($p<0.2$).

The mean 4-day parasitemia of the above 60 chicks fed the stock diet was $7289 \pm 251/10^4$ erythrocytes; the parasitemia of the corresponding 60 chicks fed the C-2 diet was 6890 ± 225. There was no significant difference between the means ($p<0.3$).

The antimalarial activity of 9 compounds was compared in chicks fed C-2 diet and stock diet (Table I). With 7 of these compounds, the difference in antimalarial activity between the 2 diets was small and probably of doubtful significance. On the other hand, sulfadiazine was at least 16 times as active in chicks fed the C-2 diet as it was in chicks fed stock diet. In preliminary trials metachloridine was about 16 times as active when used to treat chicks fed C-2 diet as it was in chicks fed stock diet.

In repeated trials, it was established that metachloridine was 16 times as active in C-2 diet-fed chicks (Table II). At the suggestion of Dr. George M. Briggs, metachloridine was tested in chicks fed an all-vegetable diet (see Methods), in which the 2 most abundant constituents were ground corn and soybean meal. Metachloridine was no more active with this diet than it was with the stock diet. Ground corn and soybean meal were tested separately as part of the C-2 diet in approximately the concentrations in which they occurred in the corn-soy diet (Table II). When 41% soybean meal (either expeller type or hexane-extracted) was incorporated in the C-2 diet, the hyperactivity of metachloridine associated with the purified diet was almost completely obliterated. Ground corn at 60% of the diet appeared to reverse partially the hyperactivity of metachloridine, but soybean meal appeared to be a richer source of inhibitor and to account for most of the inhibition of metachloridine activity observed in the all-vegetable diet.

The protein of soy at a 33% level was put into the C-2 diet at the expense of Cerelose. In chicks fed this diet, metachloridine was only slightly less active than in chicks fed the unmodified C-2 diet (Table II).

Other fractions of soybean meal were tested in single or duplicate tests (Table III). Metachloridine was as active in chicks fed 4% soybean oil in place of the same amount of corn oil of the C-2 diet, or the residue after alkaline extraction of the meal ("soybean

TABLE II. Effect of Diet on Antimalarial Activity of Metachloridine against *P. gallinaceum* Infected Chicks.

Dosage of metachloridine, mg/g b.i.d. for 4 days	Mean fourth day parasitemia (parasitized rbc/10^4 rbc)				
	Stock	C-2	C-2* 41% soybean oil meal§	C-2† 60% ground corn	C-2‡ 33% soy protein‖
.002	970 ± 106¶				
.001	2164 ± 190¶		1504 ± 297	298 ± 134	369 ± 130
.0005			2582 ± 365	1012 ± 361	402 ± 89
.00025			4008 ± 432	1786 ± 416	949 ± 183
.000125		760 ± 158**	5629 ± 501	3335 ± 490	2034 ± 462
.0000625		2150 ± 280††	6084 ± 277	4449 ± 1040	3917 ± 540
.0000313		3836 ± 350‡‡			
None	7622§§	7268 ± 596	7196 ± 236		

* 5 series, 25 chicks each dose.
† 2 ” , 10 ” ” ” .
‡ 3 ” , 15 ” ” ” .
§ Expeller type and hexane extracted, data pooled.
‖ Both alpha protein and sodium proteinate, data pooled.
¶ 22 series, 109 chicks.
** 8 ” , 40 ” .
†† 8 ” , 37 ” .
‡‡ 10 ” , 50 ” .
§§ 23 ” , 197 ” .

granules”), as it was in those fed the regular C-2 diet. The residue remaining after the acid extraction of the meal (“acid-washed grits”) appeared to have as much of the inhibitor as the soybean meal. Soybean hulls did not appear to be a rich source of the inhibitor.

Incorporation into the C-2 diet of *p*-aminobenzoic acid (100 mg/kg of diet). 5% brewer's yeast, 5% dried whole beef liver, or twice the concentration of the water-soluble vitamins of the C-2 diet, failed to inhibit the antimalarial activity of metachloridine.

Discussion. Of the 9 antimalarials tested against *P. gallinaceum* in chicks fed the C-2 diet, only sulfadiazine and metachloridine were significantly more active than in chicks fed stock diet. The hyperactivity of sulfadiazine is not surprising, since the C-2 diet has no added *p*-aminobenzoic acid and only a normal amount of folic acid.

TABLE III. Effect of Various Soy Bean Fractions, Incorporated into C-2 Diet, on Antimalarial Activity of Metachloridine against *P. gallinaceum* Infected Chicks.

Fractions of soy beans	Minimum effective dose of metachloridine, mg/g body wt
4% soy bean oil	.000125
” ” ” ”	.000125
41% ” ” hulls	.0005
” ” ” granules	.00025
” ” ” grits (acid washed)	.001

The hyperactivity of metachloridine in chicks fed the purified diet could indicate the absence from this diet of an inhibitor present in the stock diet. Such an inhibitor appears to be present in vegetable products such as soybeans and corn, though the former appears to be a richer source. The inhibitor is not *p*-aminobenzoic acid, nor does it appear to be concentrated in such sources of vitamins as dried yeast or dried liver. It is probably not one of the known water-soluble vitamins for no significant inhibition of metachloridine resulted when the vitamin content of the C-2 diet was doubled.

Neither the fatty fraction nor the protein fraction of soybeans appears to contain significant amounts of the inhibitor. The residue left after the alkaline extraction of soybean meal (“granules”) had little or no inhibitor, but the residue after acid extraction (“grits”) seemed to have significant amounts of the inhibitor.

The conclusions that can be drawn at this stage of the investigation must be tentative. The data strongly suggest that there is a substance in soybean meal which inhibits the antimalarial activity of metachloridine. This substance is neither fat nor water-soluble protein, nor is it significantly soluble in hexane. It would appear to be alkali soluble, or labile, and acid insoluble. Such a substance could conceivably be a growth factor, a natural product essential for the parasite but not for the host, and not produced in any abundance by the host. The isolation of such a substance might give some further clues as to what kind of natural products become growth factors for parasites and what kind of chemicals serve as inhibitors of such substances.

Summary. 1. The erythrocytic form of the malarial parasite *Plasmodium gallinaceum* was found to grow at a comparable rate in chicks fed either a purified diet (C-2) or a commercial stock diet. The antimalarial activity of 7 compounds was approximately the same in parasitized chicks fed either of the above diets, while sulfadiazine and metachloridine exhibited a marked increase in effectiveness in those chicks fed the C-2 diet. 2. The hyperactivity of metachloridine in chicks fed the C-2 diet was reversed almost completely when this diet contained 41% soybean meal. Neither the fatty nor protein fractions of soybeans, nor several other dietary nutrients appeared to contain significant amounts of the inhibitor.

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ERRATUM

Article on Serum Lactic Dehydrogenase . . . by Kuang-Mei Hsieh *et al.*, Vol. 89, p. 627, Tables I, II, and III, second column heading should read "mM substrate oxidized per L serum per hr."